Mucosal Adjuvant Effect of Cholera Toxin in Mice Results from Induction of T Helper 2 (Th2) Cells and IL-4[1]

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Despite pathophysiologic effects including diarrhea, cholera toxin (CT) is a potent mucosal immunogen and adjuvant. We investigated the influence of CT on T helper (Th)-type 1 (Th1) and Th2 cell-regulated Ag-specific B cell isotype and IgG subclass Ab responses elicited when the toxin was co-administered orally with different protein Ags. When mice were orally immunized with tetanus toxoid (TT) and CT as adjuvant, this regimen induced TT-specific secretory IgA responses in the gastrointestinal tract as well as serum IgG, including IgG1 and IgG2b subclasses, and IgA responses. This oral regimen also induced TT- and CT-B-specific IgE responses. In addition, CT also elicited adjuvant effects for Ag-specific IgG1, IgE, and IgA responses when two other protein Ags, OVA and hen egg white lysozyme, were given by the oral route. Quantitative reverse transcriptase-PCR was performed to assess levels of mRNA for Th1 (IFN-γ) and Th2 (IL-4) cytokine expression in TT-stimulated $CD4^+$ T cell cultures. Both Peyer's patches and splenic $CD4^+$ T cells expressed markedly increased levels of IL-4-specific message, but did not result in changes in IFN-γ mRNA expression. To determine whether the route of immunization influenced IgE responses, mice were immunized s.c. with TT and CT as adjuvant. Significant increases in total and TT-specific IgE Abs were induced when CT was co-administered. Taken together, these results show that CT acts as a mucosal adjuvant to enhance Th2-type responses and in particular, the IL-4 produced results in a characteristic Ab isotype pattern associated with this cytokine. *The Journal of Immunology,* **1995, 155: 4621–4629.**

Cholera toxin (CT),[3] a major enterotoxin produced by *Vibrio cholerae*, consists of a toxic A subunit covalently linked to a pentameter of B subunits (CT-B) which bind to mono-sialoganglioside (GM1) present on all nucleated cells but found in abundance on the lumenal surface of intestinal epithelial cells (1–5). The binding to the cell membrane induces a conformational change that allows the A subunit to penetrate into the cell and induce ADP ribosylation of adenyl cyclase regulatory protein Gs (5). The consequent increase in intracellular cAMP leads to a massive flow of water and electrolytes from epithelial cells, which gives rise to the characteristic diarrhea observed in patients with cholera. Despite these pathologic properties, CT has become the model mucosal immunogen and adjuvant. Oral administration of microgram amounts of CT induces significant secretory IgA (S-IgA) and serum IgG Abs and these Ab responses are restricted by the I-A subregion of the H-2 histocompatibility locus (6–10). Further, CT is among the few proteins that does not induce oral tolerance and can abrogate oral tolerance to unrelated proteins when simultaneously administered (11).

The ability of CT to act as a mucosal adjuvant has been confirmed by a number of investigators with a variety of Ags (11–20). To induce immunity to the target Ag, CT must be administered by the same route and at the same time (11); however, the dose of CT required for adjuvant responses may vary with different Ags. Further, CT induces long-term memory to itself (18) and to co-administered proteins (20). Some debate exists as to whether the CT-B subunit can serve as adjuvant; however, most evidence indicates that CT-B coupling to protein promotes binding to GM1 receptors and Ag uptake, while adjuvant activity requires the holotoxin (14–20). Nevertheless, the precise mechanism for the adjuvant properties of CT remains undefined.

Several studies have shown that CT can have direct effects on lymphoreticular cells and could thus provide partial explanations for adjuvant properties (21–26). For example, CT induces macrophages to produce cell-associated and secreted IL-1, enhances Ag uptake from the gut lumen, and may enhance MHC class II molecule expression and peptide presentation, although the latter point remains controversial (21–23). Further, CT facilitates B cell switching to IgA and increases the effects of IL-4 and IL-5 on IgG and IgA synthesis in LPS-triggered splenic B cell cultures (24, 25). The effect of CT on T cells is less clear; however, it has been shown that CT inhibits Con A-induced T cell proliferation and IL-2 production and was inhibitory for Th1 but not for Th2 cell clones (26). Our past studies have shown that oral immunization of mice with CT resulted in marked increases in $CD4^+$ T cells isolated from PP and SP, which preferentially secreted IL-4 and IL-5 (Th2-type cytokines) following antigenic stimulation in vitro (27,

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[3] Abbreviations used in this paper: CT, cholera toxin; CT-B, B subunit of CT; GI, gastrointestinal; GM1, mono-sialoganglioside; HEL, hen egg white lysozyme; PCA, passive cutaneous anaphylaxis; PP, Peyer's patches; RT-PCR, reverse transcriptase-PCR; S-IgA, secretory IgA; SP, spleen; TT, tetanus toxoid; rcRNA, recombinant RNA.

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28). Although our findings were consistent with the observation that the response of lamina proprial lymphocytes and PP lymphocytes to orally administered CT and keyhole limpet hemocyanin was dominated by Th2-type cytokines (29), others had suggested that oral administration of CT also induces IFN-γ production presumably by activation of Th1-type cells (30).

The development of effective mucosal vaccines requires the precise characterization of mechanisms involved in the induction of Ag-specific immune responses in mucosal and systemic tissues. In the present study, we have focused on whether orally administered CT activates Ag-specific Th2- rather than Th1-cell pathways in vivo, since conflicting views on this point have been published (26, 27, 31, 32). We provide evidence that quantitative increases in IL-4, but not IFN-γ mRNA isolated from Ag-specific $CD4^+$ Th cells directly correlate with serum IgG1 and IgE as well as with mucosal S-IgA responses.

Materials and Methods

Mice

C57BL/6 mice were obtained from the Frederick Cancer Research Facility (National Cancer Institute, Frederick, MD) at 5 to 6 wk of age. Mice were maintained in horizontal laminar flow cabinets and were free of microbial pathogens as determined by Ab screening and routine histologic analysis of organs and tissues. All mice were provided sterile food and water ad libitum. IL-4 gene disrupted mice ($IL\text{-}4^{-/-}$: 129/O1a × C57BL/6 F_2) (33) were maintained in pathogen-free conditions using a flexible Trexler isolator and individual cage microisolators in animal facilities of the University of Alabama at Birmingham Immunobiology Vaccine Center. All mice used in this study were between 8 and 13 wk of age.

Immunization

Mice were deprived of food for 2 h and given 0.5 ml of an isotonic solution containing eight parts HBSS and two parts 7.5% sodium bicarbonate by gastric intubation to neutralize stomach acidity. After 30 min, mice were orally intubated with 0.25 ml PBS containing a mixture of vaccine grade tetanus toxoid (TT) (250 μg/mouse: 250 Lf units/mg) kindly provided by Dr. Patricia J. Freda Pietrobon (Connaught Laboratories Inc., Swiftwater, PA) and CT (10 μg/mouse; List Biologic Laboratories, Campbell, CA). In some experiments, mice were orally immunized with OVA or HEL (Sigma Chemical Co.) at a dose of 5 mg/mouse together with 10 μg of CT. Groups of mice were immunized on days 0, 7, and 14 (27, 28). Other groups of mice received 0.25 ml of PBS containing TT, or OVA or HEL only. For immunization by a peripheral route, mice were immunized s.c. with TT (10 μg/mouse) and CT (1 μg/mouse), or TT (10 μg/mouse) only and boosted with the same regimen 2 wk later. Fecal extracts and serum samples were collected at weekly intervals and monitored for IgM, IgG, including all four subclasses, and IgA anti-TT, anti-OVA, or anti-HEL Ab responses (28). The sera were also monitored for total IgE and for anti-TT, anti-CT-B, anti-OVA, or anti-HEL IgE Ab responses.

Detection of Ag-specific Ab production by ELISA

Ab titers in serum and fecal extracts were determined by ELISA (28). The assay was conducted in Falcon Microtest III assay plates (Becton Dickinson, Oxnard, CA). Plates were coated with an optimal concentration of TT (100 μl of 5 μg/ml TT, equivalent to 1.25 Lf units/ml) CT-B, OVA, or HEL (100 μl of 5 μg/ml) in PBS. Plates were incubated overnight at 4°C in a humid atmosphere and washed three times with PBS. Wells were blocked with 200 μl of 10% normal goat serum in PBS containing 1% BSA for 1 h at 37°C. After washing, serial dilutions of serum or fecal extract fluid were added in duplicate and the plates were incubated for 2 h at 37°C. Serum and fecal extracts at similar dilutions from nonimmunized mice were included as controls. Following incubation, the plates were washed and a secondary Ab consisting of 100 μl of a 1:1000 dilution of biotinylated goat anti-mouse μ, γ or α heavy chain- specific Ab (Southern Biotechnology Associates (SBA), Birmingham, AL) was added to the plates. The detection enzyme consisted of a 1:2000 dilution of horseradish peroxidase-conjugated streptavidin (Life Technologies, Grand Island, NY). The plates were incubated at room temperature for 1 h and after washing, developed at room temperature with 100 μl of 1.1 mM 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid in 0.1 M citrate-phosphate buffer (pH 4.2) containing 0.01% H_2O_2. Endpoint titers were expressed as the reciprocal $\log_2$ of the last dilution which gave an optical density at 414 nm (OD_{414}) of ≥0.1 units above negative controls after a 15-min incubation. Reactions were terminated by addition of 50 μl of 10% SDS in 0.1 M citrate-phosphate buffer. For IgG subclass analysis, monoclonal biotin-labeled anti-mouse γ1, γ2a, γ2b or γ3 subclass specific Ab (PharMingen, San Diego, CA) was used exactly as described above for anti-H chain-specific analysis. The above-mentioned Abs did not exhibit any cross-reactivity as determined by reaction with monoclonal mouse IgG subclass myeloma proteins.

Detection of total serum IgE production by ELISA

Total IgE levels were determined by a modified ELISA. Nunc-Immuno-MaxiSorp plates were coated with 2 μg/ml of rat monoclonal anti-mouse IgE Ab (PharMingen) and incubated overnight at 4°C. After washing, plates were blocked with 10% rat serum diluted in PBS for 1 h at 37°C. Serial dilutions of immune serum or standard mouse IgE (PharMingen) were added in duplicate and the plates were incubated overnight at 4°C. Controls consisted of normal serum at similar dilutions. Following incubation, the plates were washed and a biotinylated rat monoclonal anti-mouse IgE Ab (PharMingen) was added to the plates (4 μg/ml). The detection enzyme consisted of 100 μl of a 1:2000 dilution of horseradish peroxidase-conjugated streptavidin (Life Technologies). The plates were incubated for 1 h at room temperature and after washing, the color reaction was developed as described above. It should be noted that a normal serum pool obtained from 40 C57BL/6 mice (10 wk of age) contained <150 ng of IgE/ml.

Detection of Ag-specific IgE Abs by passive cutaneous anaphylaxis (PCA)

The PCA was performed with Fischer rats (200–250 g) and sensitization was achieved by injecting 1:3 dilutions (beginning at 1:10) of mouse sera (0.1 ml) into the shaved back of these rats. After 16 h, the rats received an i.v. injection of 200 μg of TT, OVA, or HEL in 1 ml of 1% Evan's blue dye in PBS. To detect CT-B-specific IgE, purified CT-B was incubated with a 10-fold excess (mole/mole) of GM1 ganglioside (Sigma Chemical Co.) for 10 min at 37°C, since injection of CT-B alone would bind to ubiquitous rat GM1. The mixture, equivalent to 200 μg of CT-B (in PBS containing 1% Evan's blue dye) was injected i.v. into rats. The animals were killed 10 min after i.v. injection and the diameter of blueing resulting from the localized degranulation of mast cells was determined. The endpoint titer was selected as the last dilution resulting in a diameter of blueing of ≥5 mm.

Adsorption of serum IgE

Serum IgE was adsorbed to Polybead-hydroxylate latex microspheres (Polysciences, Inc., Warrington, PA) by the following procedure. One milliliter of a 2.5% solution of beads was centrifuged and resuspended in 7.5% sodium bicarbonate, pH 8.3. Fifty micrograms of anti-mouse IgE (PharMingen), was added to the treated beads and incubated overnight at room temperature with continuous rocking. The supernatant was assayed for protein content following centrifugation to determine the amount of Ab bound to the latex beads. Serum containing IgE Abs was adsorbed to the beads by gentle rocking at room temperature for 4 h. Following incubation, the mixture was centrifuged and the supernatant was tested for IgE by ELISA and by PCA.

Preparation of T cell suspensions from SP and PP

SP were aseptically removed and single-cell suspensions were prepared as described previously (27). PP were carefully excised from the intestinal wall and dissociated using the neutral protease Dispase (Boehringer Mannheim Corp., Indianapolis, IN) in Joklik-modified medium (Life Technologies) to obtain single cell preparations (27). Purification of $CD4^+$ T cells from PP and SP for in vitro induction of Ag-specific responses was performed by a previously described method (27). Briefly, single-cell suspensions were incubated in complete medium (RPMI 1640 supplemented with 10 ml/L of a 100X nonessential amino acids solution, 1 mM sodium pyruvate, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 40 μg/ml gentamicin, and 10% FCS) for 4 h at 37°C in 5% CO_2 to remove adherent cells. The $CD3^+$ T cell-enriched fraction was obtained by negative panning on petri plates coated with $F(ab')_2$ goat anti-mouse Ig to avoid T cell stimulation. Three rounds of panning were used to remove adherent macrophages and B cells. The $CD4^+$ T cell subset was then obtained by direct panning on plates coated with anti-L3T4 (GK 1.5) and yielded fractions of >99% $CD4^+$ T cells.

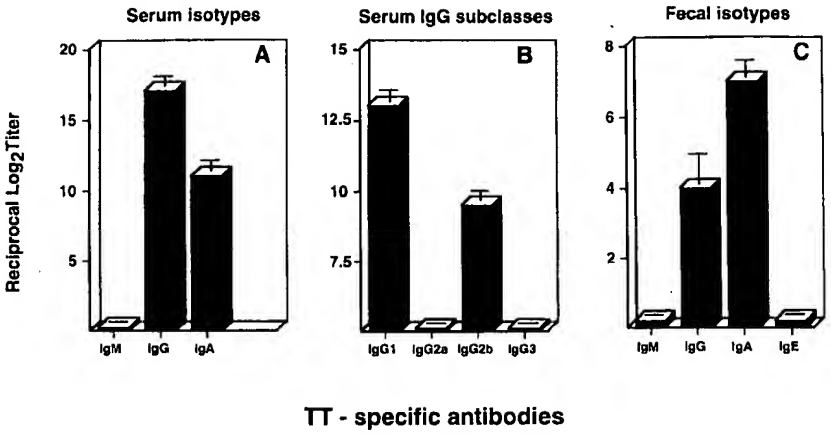


FIGURE 1. TT-specific Ab responses in mice orally immunized with TT and CT as adjuvant. The major isotypes of serum anti-TT Abs and the serum IgG subclass anti-TT titers are shown in *A* and *B*, respectively. The isotype and titers of fecal anti-TT responses are shown in *C*. The data shown are from mice 21 days following primary immunization; results are from five mice/group and are expressed as mean ± SD of five different experiments. A similar pattern but of lower magnitude for Ag-specific responses was noted when samples were obtained 14 days following primary immunization.

Ag-coated latex microspheres for induction of CD4⁺ T cell responses

TT protein was coated to latex microspheres by a modification of a previously described method (27, 34). Briefly, 0.5 ml of Polybead-hydroxylate latex microspheres (1 μm; Polysciences) were washed and resuspended in 0.1 M of borate buffer (pH 8.5). TT (400 μg) diluted in borate buffer was slowly added to the suspension and incubated overnight at room temperature with continuous rocking. After washing, the protein-coated microspheres were blocked for 30 min at room temperature in incomplete RPMI 1640 containing 1 mg/ml glycine. The suspension was then centrifuged and resuspended in RPMI 1640 containing 500 μg/ml of gentamicin to insure sterility. For analysis of Ag-specific Th1 and Th2 cell responses, SP and PP $CD4^+$ T cells were cultured at a density of 1×10^6 cells/ml in the presence of TT-coated latex microspheres (at a bead-to-cell ratio of 10:1) and T cell-depleted, irradiated (3,000 rad) splenic feeder cells (1×10^6 cells/ml) and IL-2 (10 U/ml) (27). Ag-stimulated T cells were harvested at appropriate intervals for analysis of mRNA expression (see below).

Cytokine-specific PCR analysis

For the detection of IFN-γ, IL-2, IL-4, IL-5, and IL-6 specific mRNA in $CD4^+$ T cells, a standard RT-PCR amplification protocol was employed (27, 35) and modified as previously described (27, 36). For the isolation of RNA, the method of acid guanidium thiocyanate phenol chloroform extraction procedure was used (37). RNA was extracted and was then subjected to the cytokine-specific RT-PCR using 2.5 U/μl Superscript II Reverse Transcriptase (Life Technologies). PCR products separated by electrophoresis in 2% agarose gels were stained with ethidium bromide (0.5 μg/ml) and visualized under UV light.

For quantitation of IFN-γ and IL-4-specific mRNA, a RT-PCR was adopted using recombinant mRNA (rcRNA) as internal standard (38). We constructed a connected rcRNA for IFN-γ, IL-4, and β-actin as described by others (39). The PCR reaction was conducted in 50 μl of PCR buffer, 3 mM $MgCl_2$, 0.2 mM of each dNTPs, 30 pmol of rcRNA forward and reverse primers for IFN-γ, IL-4, and β-actin, 200 ng genomic DNA (spacer gene) and 2.5 units *Taq* polymerase (Perkin-Elmer Cetus, Foster City, CA) at 85°C. After heating at 94°C for 3 min, samples were cycled 30 times with a 30-s denaturing step (94°C), a second annealing process (59°C), and a 45-s extension step (72°C) in a thermal cycler (Perkin-Elmer Cetus). A 5-min extension step (72°C) was performed at the end of the procedure. The PCR products were purified with the Wizard PCR Preps DNA Purification System (Promega Corp., Madison, WI). After construction of this synthetic gene containing IFN-γ, IL-4, and β-actin, the PCR product was inserted into a pGEM-T Vector containing the T7 polymerase promotor (Promega). Transformation was performed by using *Escherichia coli* JM109. To obtain rcRNA, a purified synthetic gene was linearized by Spe I (Promega) and in vitro transcription was then performed by the Riboprobe Gemini System using T7 RNA polymerase (Promega). To obtain purified rcRNA, the transcripts were treated with RQI DNase (Promega) and then further purified by using Oligotex-dT latex particles (Oligotex-dT mRNA kits, Qiagen, Chatsworth, CA).

For competitive RT-PCR, total RNA was run with cytokine-specific rcRNA as a competitive template. Aliquots of total RNA (8–630 ng for IFN-γ, 11–900 ng for IL-4, and 0.19–15 pg for β-actin) were spiked with a series of diluted rcRNA internal standards. A standard RT-PCR was then performed. Quantitation was achieved by addition of 5 μCi of [^{32}P]dCTP (Amersham, Arlington Heights, IL) to the PCR reaction mixture (40). Finally, the PCR products were electrophoresed as described above. The [^{32}P]dCTP content of IFN-γ, IL-4, and β-actin-specific bands was determined by liquid scintillation counting.

Statistical analysis

Results are reported as mean ± SD. Statistical differences between paired mean values were evaluated by Student's *t*-test.

Results

Ag-specific IgG subclasses and IgA

It is now established that oral administration of small amounts of CT with soluble proteins results in significant mucosal and serum Ab responses. We have assessed serum IgG subclass and mucosal Ab isotypes to this regimen during peak responses in C57BL/6 mice. As one might expect, serum Abs to TT were mainly of the IgG isotype followed by IgA; no IgM responses were evident (Fig. 1*A*). When serum IgG subclass responses to TT were assessed, the major subclass was IgG1 followed by IgG2b. No serum IgG2a or IgG3 Abs were detected to TT in orally immunized mice (Fig. 1*B*). Further, mucosal IgA Abs were present in fecal extracts, with somewhat lower titers of IgG (Fig. 1*C*). When TT was given alone via the oral route to C57BL/6 mice, low or undetectable levels of Ag-specific serum IgG responses were seen (e.g., the following reciprocal $\log_2$ titers were seen: IgG1, 9; IgG2a, 6; IgG2b, 7 and IgG3 < 6) and no Ag-specific S-IgA was detected in fecal extracts (data not shown). These results support the concept that oral administration of CT as adjuvant results in Th2-type responses, since Th2 cell-secreted cytokines have been shown to support IgG1 and IgA synthesis.

CT-induced IgE

Strong support for Th2 cell regulation of Ab responses following oral administration of TT and CT was provided by the finding that immune sera contained elevated levels of IgE (Fig. 2*A*). We have established that naive C57BL/6 mice do not produce detectable levels of IgE (<150 ng/ml) under the housing conditions employed in our studies. We have determined the kinetics of IgE Abs in mice orally immunized with TT and CT as adjuvant. Interestingly, no increases in serum IgE were detected 1 wk following a single oral dose; however, peak IgE levels were noted after two oral doses spaced at weekly intervals and lower levels were observed after three oral doses (Fig. 2*A*). The elevation of serum IgE was not seen in mice orally immunized with TT only (data not shown).

We assessed TT-specific IgE Abs by using the PCA assay in rats (Fig. 2, *B* and *C*). Our results showed that sera of mice orally

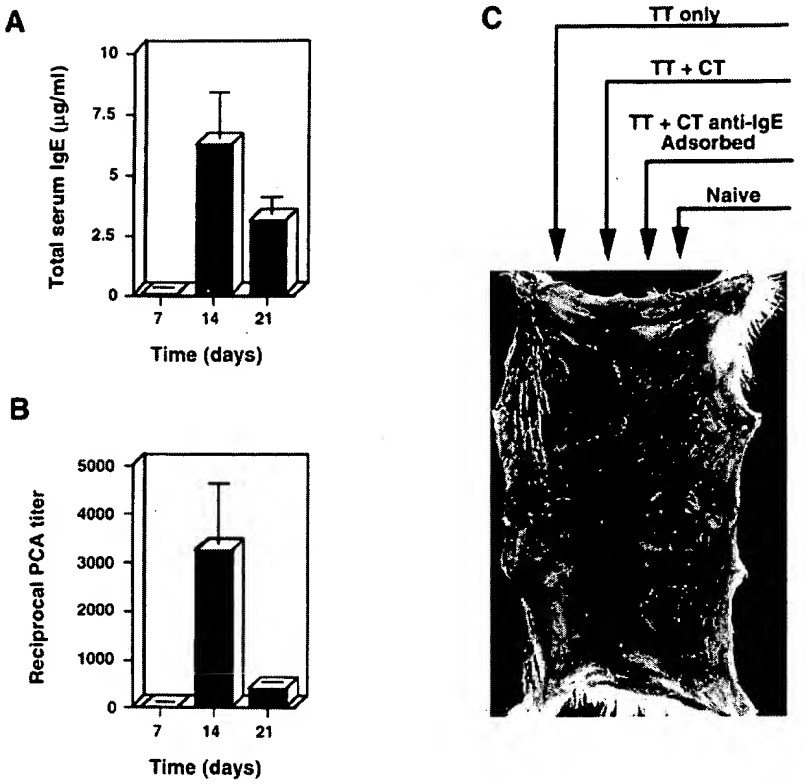


FIGURE 2. Serum IgE responses in mice orally immunized with TT and CT as adjuvant. The kinetics of total serum IgE (*A*) and TT-specific IgE (*B*) responses are shown. *C* illustrates a typical PCA reaction for TT-specific IgE. Serum IgE levels from a pool of 40 naive mice were ≤150 ng/ml. Results are from five mice/group and are expressed as mean ± SD of four different experiments (*A* and *B*).

Table I. *IgE response induced in mice orally immunized with TT and CT as adjuvant*[a]

Serum	Total IgE (ng/ml)	Ag used in the PCA	Reciprocal PCA Titer
Naive	<150	TT	—[c]
TT + CT	10,250	TT	2,430
TT + CT	10,250	CT-B + GM1	830
Absorbed[b]	<150	TT	—[c]

[a] Results shown are representative of four different experiments performed on sera taken during the optimal IgE response (day 14). Total and Ag-specific IgE was determined as described in *Materials and Methods*.

[b] Absorption with monoclonal anti-IgE Abs was performed on the same day 14 sera.

[c] Undetectable by PCA.

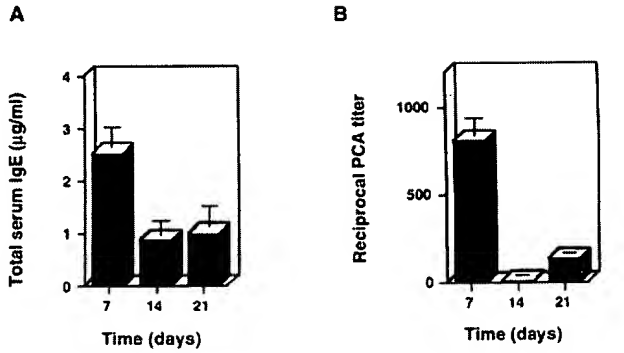


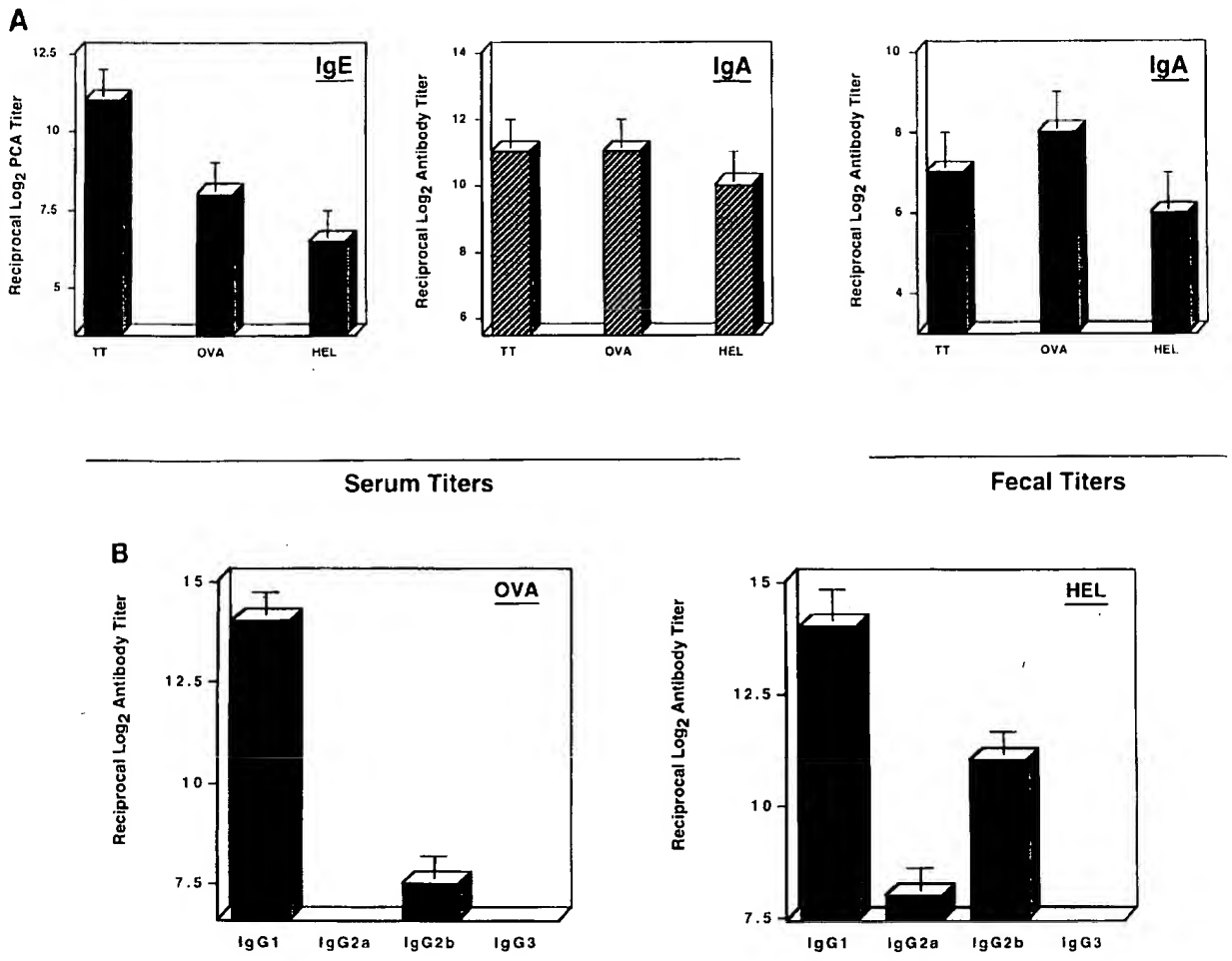
FIGURE 3. Serum IgE responses in mice immunized s.c. with TT and CT as adjuvant. The kinetics of total serum IgE (*A*) and TT-specific IgE (*B*) Ab responses are shown. Results are from five mice/group and are expressed as the mean ± SD of three different experiments.

immunized with TT and CT as adjuvant contained IgE anti-TT Abs (Fig. 2, *B* and *C*). In this analysis, no IgE anti-TT Abs were detected 1 wk after oral immunization; however, maximum IgE Abs were present after two oral doses (Fig. 2*B*) at the same interval in which maximum total IgE levels were noted (Fig. 2*A*). When serum samples from mice orally immunized with TT only were examined by the PCA assay, no Ag-specific IgE response was detected (Fig. 2*C*). It was important to establish that the PCA assay was specific for IgE. When immune mouse sera was adsorbed with monoclonal anti-IgE Abs, the total IgE Abs were reduced to levels below detection by ELISA (Table I). Further, this absorption removed IgE anti-TT Abs as determined by PCA (Table I and Fig. 2*C*). To determine whether IgE Abs to CT were also induced, the PCA test was performed with CT-B bound to GM1 and significant titers of IgE anti-CT-B were detected (Table I). Taken together, the data support the concept that, in mice orally immunized with CT, T cells provide a Th2-type help with subsequent production of IgE Abs.

In a recent study, it was proposed that CT can sensitize for a subsequent anaphylactic IgE response only when mice are orally immunized with the sensitizing Ag and CT (31). In fact, mice systemically immunized with the Ag alone underwent anaphylactic reactions in the absence of CT (31). Thus, in our study, it was important to determine whether the Ag itself could induce an IgE response in the complete absence of CT. To address this point, groups of mice were immunized s.c. with TT alone or with TT and CT as adjuvant. It was interesting to note that only the latter regimen induced elevated levels of serum IgE (Fig. 3*A*) and TT-specific IgE Abs (Fig. 3*B*). On the other hand, mice receiving TT alone did not exhibit any detectable total or TT-specific IgE Abs (data not shown). Taken together, these results show that the IgE responses observed were due to CT regardless of the route of immunization used.

Ab isotype responses to OVA and HEL

To ensure that the isotype and subclass profiles observed following oral immunization with TT and CT as adjuvant were not restricted



IgG Subclass Antibody Titers

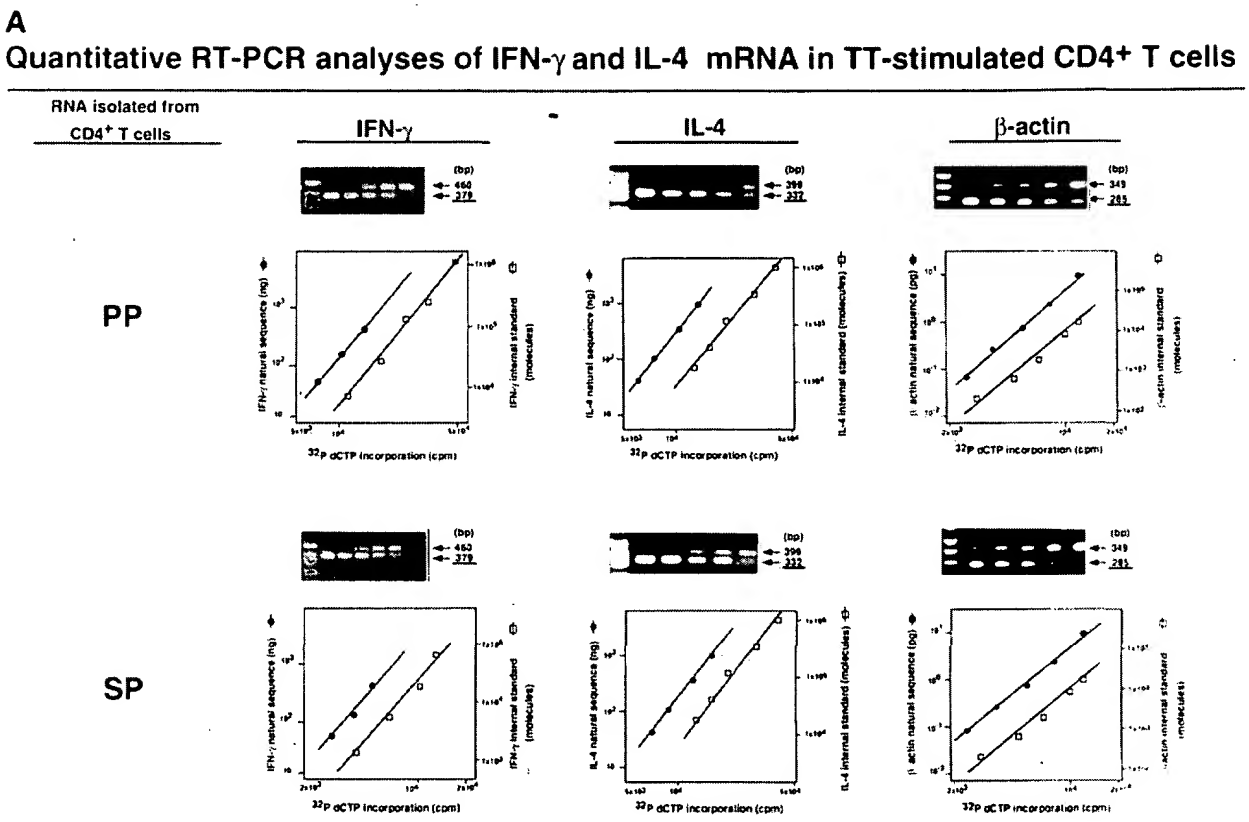
FIGURE 4. CT induces Ag-specific IgE responses in mice orally immunized with OVA or HEL. Mice were given OVA (5 mg) or HEL (5 mg) in the presence of CT (10 μg) by gastric intubation. Serum and fecal samples were analyzed at the peak of the Ab response. These results are based on the studies of five mice/group and are expressed as mean ± SD of three different experiments.

to certain Ags, two other proteins, OVA and HEL, were also tested as oral immunogens together with CT as adjuvant (Fig. 4). Both OVA and HEL-specific serum IgE responses were induced following oral administration (Fig. 4*A*). Further, significant Ag-specific serum and mucosal IgA Abs were noted. When serum IgG subclass anti-OVA and anti-HEL responses were assessed, the major response was IgG1 followed by IgG2b (Fig. 4*B*). These results clearly show that similar patterns of Ag-specific Ab responses are induced following oral administration of different protein Ags when CT is used as adjuvant.

Analysis of cytokine-specific mRNA in TT-specific CD4$^+$ T cells

It was important to clearly establish that CT acts as an adjuvant through induction of Th2-type cytokines. Purified CD4$^+$ T cells from both PP and SP of mice orally immunized with TT and CT were restimulated in vitro with TT to assess Th1 and Th2 cytokine production and mRNA levels. In this analysis, IL-4, IL-5, and IL-6 mRNAs were present, while IFN-γ and IL-2 mRNAs were identical to those present in unstimulated CD4$^+$ T cell cultures. These results were essentially identical to those published earlier (27) and are not presented here. Analysis of cytokine production by ELISPOT revealed that high numbers of IL-4, IL-5, and IL-6-producing CD4$^+$ T cells were present, while IFN-γ and IL-2 spot-forming cells (SFC) were the same as control cultures (27; and data not shown).

We employed a quantitative RT-PCR to assess levels of IFN-γ and IL-4 message in TT-induced CD4$^+$ T cell cultures as examples of Th1 and Th2 cytokines, respectively. Both PP and SP CD4$^+$ Th cells triggered with TT expressed markedly increased levels of IL-4 specific mRNA, when compared with unstimulated CD4$^+$ T cells or TT-treated CD4$^+$ T cells from unimmunized mice (Fig. 5, *A* and *B*). A similar increase in IL-4 mRNA was seen in CT-B-stimulated CD4$^+$ T cell cultures from mice orally immunized with TT and CT as adjuvant (data not shown). In contrast, the levels of IFN-γ mRNA between Ag-stimulated and unstimulated CD4$^+$ T



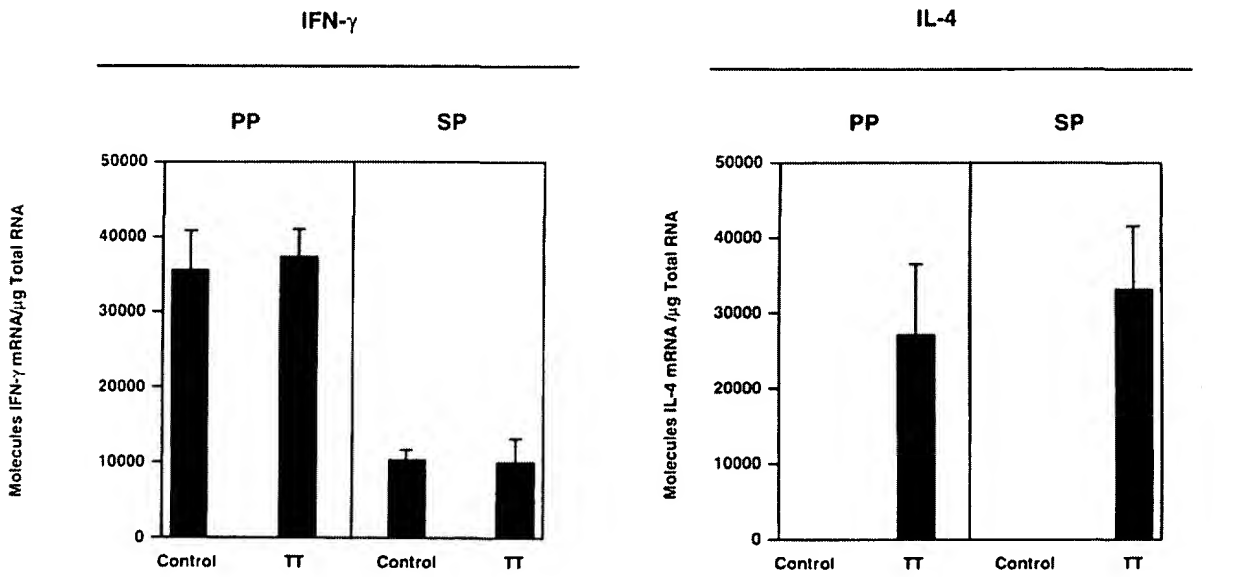


FIGURE 5. Analysis of IFN-γ and IL-4 mRNA in TT-stimulated PP and SP CD4+ T cells by quantitative RT-PCR. Pictures in Figure 5*A* show IFN-γ, IL-4, and β-actin PCR products separated on a 2% agarose gel. Underlined bp represent an internal control. The six graphs depict results obtained by quantitation of ^{32}P radioactivity. Figure 5*B* shows the amount of IFN-γ and IL-4 specific mRNA in either TT-stimulated PP or SP or control cultures (e.g., unstimulated and stimulated with OVA). Results are from five mice/group and are expressed as mean ± SD of three different experiments.

Table II. *Lack of TT-specific IgG1 and IgE responses in IL-4$^{-/-}$ mice orally immunized with TT and CT*[a]

		TT-Specific Immune Responses		
		Reciprocal $\log_2$ titer		Reciprocal PCA titer
Mouse Strain	Vaccine	IgG1	IgG2a	IgE
IL-4$^{-/-}$	TT + CT	—[b]	10 ± 6	—[b]
IL-4$^{-/-}$	TT only	—	—	—
(129 × B6) F_2	TT + CT	12.8 ± 1.4	—[b]	2,430
(129 × B6) F_2	TT only	—	—	—

[a] IL-4$^{-/-}$ and background IL-4$^{+/+}$ (129 × B6 F_2) mice were orally-immunized with TT (250 μg) and CT (10 μg) on three occasions at weekly intervals.
[b] Undetectable by ELISA or by PCA.

cells isolated from PP and SP of mice orally immunized with TT and CT were essentially identical (Fig. 5*B*). These findings provided further evidence that CT can activate Ag-specific Th2 cytokine pathways (e.g., IL-4) rather than Th1 (e.g., IFN-γ) responses.

TT-specific Ab responses in IL-4$^{-/-}$ knockout mice orally immunized with TT and CT

To test the possibility that the mode of CT adjuvanticity is the activation of Th2-type cells in vivo, we have taken advantage of IL-4$^{-/-}$ mice that lack Th2-type responses (33). To this end, IL-4$^{-/-}$ mice were orally immunized with TT and CT using an identical immunization regimen to that described above. As expected, TT-specific IgE responses were not induced in IL-4$^{-/-}$ mice orally immunized with TT and CT (Table II), while oral immunization of wild-type mice of the same background (129/O1a × C57BL/6 F_2) resulted in the induction of high serum IgE anti-TT Ab responses. When Ag-specific IgG subclass responses were examined in serum from orally immunized IL-4$^{-/-}$ mice, no Ag-specific IgG1 responses were seen, while IgG2a Abs were detected (Table II). Thus, it is likely that the absence of IL-4 allows IFN-γ to be produced for support of compensatory IgG2a Ab responses. On the other hand, normal F_2 mice gave high TT-specific IgG1, but not IgG2a Ab responses. The titers of IgG1 anti-TT Abs in the F_2 background mice were comparable to those of C57BL/6 mice (Fig. 1*B* and Table II). Oral immunization of wild-type or IL-4$^{-/-}$ mice with TT only did not result in detectable TT-specific IgG1, IgG2a, or IgE Ab responses (Table II). These findings confirmed that IL-4 and Th2-type responses are involved in and are necessary for the mucosal adjuvanticity of CT.

Discussion

The mucous membranes are portals of entry for most bacterial and viral pathogens and major efforts are underway to develop vaccines for provision of effective mucosal immunity (reviewed in Ref. 41). In this regard, the induction of significant Ag-specific mucosal S-IgA Abs in the gastrointestinal (GI) tract is often difficult to achieve by oral administration of protein-based vaccines. A major exception to weak immunogenicity to orally administered proteins is CT, which is both an effective mucosal immunogen and adjuvant. Even though the precise mechanisms of CT adjuvanticity had until now remained unclear, numerous studies have shown that CT induced brisk mucosal S-IgA Abs in addition to elevated serum IgG and IgA Ab responses. We have now provided compelling evidence that the Ab patterns induced in vivo by CT directly correlate with the Th2-type phenotype expressed in vitro by CD4$^+$ Th cells.

One major aim of this study was to characterize the precise nature of the Ab response induced in mice following oral administration of the protein vaccine TT together with CT as an adjuvant. Oral administration of TT and CT in three oral doses given at weekly intervals gave significant endpoint titers of TT-specific IgG and IgA Abs in sera as well as S-IgA Abs in fecal extracts, a finding common to most previous studies with CT (11–20, 27, 28). However, while investigating the effect of this oral regimen on the total serum Ig isotypes, we noted that total IgE levels increased 40-to 60-fold (Fig. 2*A*). Further, careful analysis of total IgG subclasses also revealed that two- to three-fold increases in total serum IgG1 occurs in mice given oral TT and CT (data not shown). These analyses of isotype and subclass of Ag-specific Ab and total Ig production in mice given CT has allowed us to suggest that this mucosal adjuvant up regulates Th2 type CD4$^+$ T cell responses in vivo.

It is known that cytokines play a major role in selecting the isotype of Ab produced during the immune response. For example, treatment of mice with monoclonal anti-IL-4 or anti-IL-4 receptor (43, 44) and the use of IL-4-deficient mice (32, 33, 45) have provided evidence that IgE responses can be down-regulated or even completely inhibited. In addition, although no single cytokine appears to be required for the generation of IgG1 or IgG2a responses in vivo, IFN-γ has been shown to enhance IgG2a production, while IL-4 stimulates IgG1 responses (46–48). Elegant studies by others (49, 50) have shown the existence of two subsets of murine CD4$^+$ T cells, i.e., Th1 cells that produce IFN-γ, IL-2 and TNF-β, and Th2 cells that secrete IL-4, IL-5, IL-6, and IL-10 (49, 50). It is clear that in the murine system, an immune stimulus activating Th2 cells would be expected to induce Ab responses characterized by production of IgG1 and IgE, while a stimulus that results in activation of Th1 cells would generate a predominant IgG2a response. Although our previous studies (27) indicated the existence of a Th2-type profile in vitro in mice orally immunized with TT and CT as an adjuvant, the consequences of this Th2 type response in vivo was not assessed. Analysis of Ag-specific Ab responses in the present study revealed that significant levels of TT-specific serum IgG1, IgG2b, IgA, and IgE Abs were induced while TT-specific IgM, IgG2a, and IgG3 were undetectable (Figs. 1 and 2B). Further, we used a quantitative RT-PCR to assess levels of mRNA for IL-4 in Ag-stimulated CD4$^+$ T cell cultures. Marked increases in IL-4-specific mRNA levels were noted in Ag-induced CD4$^+$ T cell cultures from these mice (Fig. 5*B*). On the other hand, the level of IFN-γ message, an indication of Th1-type responses, did not change. The Ig isotype/subclass profiles for anti-TT Abs observed in vivo were consistent with the Th2-type cytokine profile obtained by quantitative analysis of cytokine message in these Ag-triggered CD4$^+$ T cell cultures. Further, as one might expect, TT-specific IgG1 (but not IgG2a) and IgE responses were not induced in IL-4$^{-/-}$ mice orally immunized with TT and CT. In this regard, it has been shown that IL-4$^{-/-}$ mice lack Th2-type cell responses (33) and it is likely that the absence of IL-4 allows IFN-γ to be produced for support compensatory IgG2a Ab responses.

Other studies from our group have shown that intranasal immunization of mice with large doses of TT and CT induced an anaphylactic response following a second immunization (J. Simecka, M. Marinaro, R. J. Jackson, and J. R. McGhee, manuscript in preparation); however, if the dose was reduced, mice survived a second and even a third immunization. In this regard, others have shown that following oral immunization with HEL and CT, mice of H-2^k and H-2^q haplotypes underwent a fatal anaphylactic response after i.p. challenge with Ag, while mice bearing the H-2^a (B10.A) haplotype survived (31). It should be noted that some of the mice used in that study were high responders to HEL and when the serum Ag-specific IgG subclasses (IgG1 and IgG2a) were evaluated, the IgG1 titers were significantly different from those obtained in our

study (31). Moreover, Ab isotypes were not evaluated in mice surviving the i.p. challenge (B10.A mice). The reasons for these apparent discrepancies in Ab responses are not easily explained.

In the present study, we used HEL and OVA and when these protein Ags were co-administered with CT via the oral route, a similar profile to that obtained with TT and CT immunized mice was noted. Thus anti-OVA or anti-HEL serum IgG1 and IgE as well as S-IgA in fecal samples were seen (Fig. 4, *A* and *B*). When these mice were systemically challenged with homologous Ag at the time of peak IgE response (day 14) and 3 wk after the last immunization, none of the mice demonstrated signs of anaphylaxis (data not shown). Moreover, mice orally immunized with TT and CT, that were challenged s.c. with 100 minimum lethal doses of tetanus toxin not only survived the challenge, but did not exhibit anaphylaxis (28). Since high levels of Ag-specific IgG and IgA Abs were also induced in serum of these mice, these two Ab isotypes may block binding of the Ag to IgE on sensitized mast cells. In our studies, the adsorption of IgE from sera of mice immunized with TT and CT ruled out any role for IgG subclass reaginic Abs contributing to the PCA reaction (Table I and Fig. 2*C*). Thus, we have provided direct evidence for the presence of biologically active TT-specific IgE Abs. Furthermore, the failure to elicit an anaphylactic response when mice were orally boosted on day 14 (at the peak of the IgE response) may be again related to the high titers of Ag-specific Abs present in the circulation. Thus, these results imply that IgG1 Abs that have limited capacity to react with cells and that molecules involved in the inflammatory process may be better adapted to providing effector functions such as neutralization of exotoxins. Indeed, our analysis of Ag-specific IgG subclasses revealed that IgG1 accounted for the majority of the serum IgG Ab response (Figs. 1*B* and 4*B*). We have shown that IgE responses were induced only in the presence of CT either in mice immunized by the oral or by the s.c. route. The latter finding is in contrast to the findings of others (31). In those studies, it has been proposed that CT sensitized only mice orally immunized for the subsequent systemic challenge with the Ag, since systemic immunization with the Ag alone (in the absence of CT) could also sensitize mice for anaphylactic IgE responses. Thus, the authors concluded that sensitization was more likely due to the nature of the Ag in the context of a particular MHC haplotype (31). We have provided evidence that the presence of CT is required for the induction of an IgE response and that this can occur regardless of the route of immunization.

Past studies of others have suggested that the response to Ags co-administered with CT is not related to the immune response to the toxin itself (51). In our studies, by immunizing the same strain of mice (C57BL/6 of the H-2^b haplotype) with different protein Ags (TT, HEL, OVA) we have addressed the question of whether the Th2-type response observed was characteristic of the particular Ag chosen (TT). Our findings clearly show that the mucosal adjuvant CT induces Th2-type responses with subsequent production of IgE to all three Ags tested. In support of this, BALB/c mice (H-2^d haplotype) immunized with TT also exhibited Th2-type responses and IgE Abs only in the presence of CT when administered by the intranasal route (J. Simecka, M. Marinaro, R. J. Jackson, and J. R. McGhee, manuscript in preparation). Thus, the use of different Ags and different strains of mice allowed us to conclude that CT exerts its mucosal adjuvant activity through a pathway involving IL-4 and Th2-type responses. The lack of mucosal adjuvanticity in IL-$4^{-/-}$ mice supports our conclusion (32).

Although it has been suggested that IL-$4^{-/-}$ mice failed to induce mucosal immune responses to Ags delivered orally with CT due to a failure to induce germinal center reactions in the PP (32), we support the idea that lack of Th2-type responses and IL-4 in IL-$4^{-/-}$ mice is more likely responsible for the loss of mucosal adjuvanticity of CT. The precise molecular mechanisms underlying the activation of Th2-type cells by CT in vivo is currently under investigation in our group. It would be interesting to propose that CT may specifically activate the promotor region for Th2 cytokines including IL-4, IL-5, and IL-6. To this end, it was shown that PGE_2, like CT, can enhance cytoplasmic cAMP for subsequent induction of Th2, but not Th1 cytokines (52). It should also be noted that pertussis toxin (PT) can also up-regulate IL-4 responses in SP and mesenteric lymph nodes of mice immunized with OVA, and this up-regulation was associated with increased levels of IgE (53). It is possible that both CT and PT may activate signal transduction pathways for Th2-type cytokines such as IL-4 via ADP ribosylation of G proteins. Current studies in our laboratory are addressing these possibilities.

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Regulation of Mucosal and Systemic Antibody Responses by T Helper Cell Subsets, Macrophages, and Derived Cytokines Following Oral Immunization with Live Recombinant *Salmonella*[1]

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We have assessed regulatory Th cell and cytokine responses in mice after oral immunization with recombinant *Salmonella* (BRD 847) expressing fragment C of tetanus toxoid, since little information is available to explain how these vectors induce mucosal IgA responses. A single dose of BRD 847 elicited serum IgG2a and mucosal IgA anti-tetanus toxoid Ab responses. To assess Th1- and Th2-type responses, $CD4^+$ T cells from Peyer's patches and spleen were restimulated in vitro, and cytokine-specific ELISPOT, ELISA, and reverse transcriptase-PCR assays were used to assess cytokine patterns. $CD4^+$ T cells produced IFN-γ and IL-2 as well as IL-10, but not IL-4 or IL-5. Although IL-6 was elevated, further purification of cells from in vitro cultures into $CD4^+$ $Mac\text{-}1^-$ T cells and $Mac\text{-}1^+$ $CD4^-$ cells revealed that only the latter cell population had consistently elevated IL-6 gene expression, whereas both sorted populations exhibited increased IFN-γ and IL-10 gene expression. Thus, orally administered recombinant *Salmonella* expressing fragment C of tetanus toxoid elicited dominant Ag-specific Th1-type responses together with Th2-type cells producing IL-10 in both mucosal and systemic tissues. Macrophages producing IL-6 were also evident. Our results are consistent with the suggestion that Ag-specific Th1 cells and their derived cytokines, IFN-γ and IL-2, and Th2-derived IL-10 together with IL-6 produced by macrophages provide important signals for the development of mucosal IgA and serum IgG subclass responses in the absence of preferential expression of Th2 cytokines IL-4 and IL-5. *The Journal of Immunology,* **1996, 156: 1504–1514.**

Attenuated avirulent *Salmonella* strains have received considerable attention as mucosal vaccine delivery vectors for recombinant proteins associated with virulence (1–4). Following oral administration, *Salmonella* replicate directly in the mucosa-associated tissues (e.g., Peyer's patches (PP)[3]) and thereafter disseminate via the mesenteric lymph node (MLN) to systemic sites (e.g., spleen (SP)). This characteristic dissemination pattern, i.e., growth in mucosal and systemic sites, allows *Salmonella* to induce broad-based immune responses, including cell-mediated, humoral, and secretory IgA Ab responses. Although a large number of genes from bacteria, viruses, parasites, and mammalian species have been expressed in attenuated *Salmonella* (2), few studies have fully characterized both T and B cell responses to the expressed protein Ag. In particular, the balance between Ag-specific, $CD4^+$ Th1 and Th2 cells and their subsequent influence on subclass-specific IgG and mucosal IgA responses has received little attention in these systems. Such clarity is paramount to the development of delivery protocols that will provide the appropriate immune response to a given pathogen. Indeed, the cytokines produced by Ag-specific Th cells are in part responsible for the type of effector immune responses induced. Two major $CD4^+$ T cell subsets have been described, as determined by the pattern of cytokines they produce; Th1 cells secrete IFN-γ, IL-2, and TNF-β, whereas Th2 cells produce IL-4, IL-5, IL-6, and IL-10 (5, 6). Th1 cells are efficient inducers of cell-mediated immunity, including activation of macrophages (Mϕ) and CTL, while Th2 cells provide better help for B cell responses, including those of IgG1, IgE, and IgA isotypes.

Salmonella typhimurium, like other intracellular microorganisms, i.e., *Leishmania major*, *Listeria monocytogenes*, and *Mycobacterium tuberculosis*, have been shown to induce a Th1-dependent immune response (7–12). Further, IFN-γ is essential for clearing *S. typhimurium* infections in vivo, in part through the activation of Mϕ for intracellular killing (12–15). In this regard, SP, PP, and MLN cells from mice orally infected with *S. typhimurium* produced elevated levels of IFN-γ upon restimulation in vitro with killed *Salmonella* (14). In another study, when SP cells

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[3] Abbreviations used in this paper: PP, Peyer's patch; MLN, mesenteric lymph node; SP, spleen; Tox C, fragment C of tetanus toxin; TT, tetanus toxoid; CT, cholera toxin; DTH, delayed-type hypersensitivity; PBS-T, PBS-0.05% Tween; ELISPOT, enzyme-linked immunospot assay; SFC, spot-forming cells; RT-PCR, reverse transcriptase-PCR; GI, gastrointestinal.

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of mice orally immunized with *S. typhimurium* expressing gp63 surface protein of *Leishmania major* were restimulated with the recombinant protein, supernatants contained increased levels of IFN-γ, while IL-4 production was suppressed (16). However, in both studies, whole SP cells rather than purified $CD4^+$ T cells were assayed. Nonetheless, it appears that oral administration of recombinant (r) *S. typhimurium* expressing protein Ag generates protein-specific Th1-type responses in systemic and possibly in mucosal tissues. Although both Th1 and Th2 cells may regulate IgA expression, the latter Th cell subset has been shown to be more efficient (17). Th2 cell-derived cytokines, including IL-5, IL-6, and IL-10, are considered major soluble factors that support and enhance IgA responses (18–21). These observations together with the capacity of *Salmonella* vectors to potentiate IgA responses to expressed proteins (3) raises the important question of what type of Th cell regulates mucosal IgA immunity in these systems. Thus, development of *Salmonella*-based vectors that provide specific types of immune responses to foreign Ag will require a more thorough investigation of Ag-specific Th1 and Th2 cell responses for systemic Ig isotypes as well as mucosal secretory IgA responses.

An attenuated r*S. typhimurium* strain (BRD 847) that expresses fragment C (Tox C) of tetanus toxin (TT) was shown to induce long-lasting immune responses and protection against systemic lethal tetanus toxin challenge in mice following a single oral dose of 10^{10} CFU (22). Plasmid stability and, thus, long term Tox C expression were based on the use of an anaerobically inducible promotor (*nirB*) in plasmid pTET*nir*15 (22). We used BRD 847 as a model bacterial vector for the assessment of Th1, Th2, and B cell responses to the orally delivered Tox C. To provide direct comparison of immune responses, we included a second vaccine regimen, TT co-administered with the mucosal adjuvant cholera toxin (CT). This second protocol has been shown to elicit dramatic Ag-specific Th2 cell responses characterized by the induction of IL-4 and IL-5 in conjunction with systemic IgG1 and IgE and mucosal IgA Abs (23–26). These two distinct protocols for mucosal prototype vaccine delivery enabled us to gain insight into Th1 and Th2 cell induction and cytokine regulation of mucosal IgA responses to the vaccine TT.

Materials and Methods

Mice

C57BL/6 and BALB/c mice were obtained from the Frederick Cancer Research Facility (National Cancer Institute, Frederick, MD). Mice were maintained in horizontal laminar flow cabinets and were free of microbial pathogens, as determined by Ab screening and tissue histopathology. All mice used in this study received sterile food and water *ad libitum*, and were between 8 and 16 wk of age when used for these experiments.

Recombinant bacteria, vaccines, and adjuvant

Detailed characteristics of r*S. typhimurium* (*aroA*$^-$, *aroD*$^-$) strains BRD 509, BRD 753, and BRD 847 used in this study were described previously (22). BRD 753 and BRD 847 were constructed by introducing plasmids that express the nontoxic immunogenic fragment C of TT into the live oral vaccine strain BRD 509. BRD 753 harbors an unstable plasmid, pTET85, and constitutively expresses fragment C from the *tac* promoter, whereas BRD 847 contains a highly stable plasmid, pTET*nir*15, and expresses fragment C under anaerobic conditions from the *nir*B promotor. Purified TT was kindly provided by Dr. Patricia J. Freda Pietrobon (Connaught Laboratories, Inc., Swiftwater, PA). CT was purchased from Sigma Chemical Co. (St. Louis, MO).

[4] Okahashi, N., M. Yamamoto, J. L. VanCott, S. N. Chatfield, M. Roberts, H. Bluethmann, T. Hiroi, H. Kiyono, and J. R. McGhee. 1996. Oral immunization of IL-4 knockout mice with *Salmonella* or cholera toxin reveals that $CD4^+$ Th2 cells producing IL-6 and IL-10 are associated with mucosal IgA responses. *Submitted for publication.*

Immunizations

Salmonella were grown statically for 14 h at 37°C in 500 ml of L-broth with or without ampicillin (100 μg/ml) as described previously (22). Groups of five C57BL/6 or BALB/c mice were given a single oral dose of 5×10^9 CFU (200 μl/mouse) by gastric intubation and killed on various days for up to 8 wk. Similar oral inoculum doses were previously shown to induce protective Ab responses in BALB/c mice against systemic lethal TT challenge (22). Colonization of orally administered r*Salmonella* was confirmed by plating serial dilutions of dissociated tissue preparations from PP and SP of immunized mice onto L-agar plates (27). Because IgG subclass and Th cell responses were not altered following a boost on day 28, only data following a single dose is shown. For comparative purposes, a second vaccine regimen that has been shown to induce Ag-specific Th2 cell and IgA B cell responses (23–25, 28, 29) was included. In this regard, mice were given three doses of TT (250 μg) and CT (10 μg) at 0, 7, and 14 days as previously described (28, 29) and were killed on day 28. Blood and fecal samples were collected from these different groups of mice at weekly intervals for Ab isotype analysis.

Measure of delayed-type hypersensitivity (DTH) responses

Cell-mediated immune or DTH responses were measured in vivo 6 wk after oral immunization of mice with BRD 509 (no expression of Tox C), BRD 753 (unstable expression), and BRD 847 (stable expression) using a standard procedure (30). Thirty micrograms of TT in 10 μl sterile PBS was injected into the left ear pinna; the other ear pinna received sterile PBS as a control. Ear swelling was measured 24 h later with a spring-loaded dial thickness gauge (Young & Van, Birmingham, AL). The DTH response was expressed as the difference (in millimeters) in ear swelling between the TT- and PBS-injected ears.

Measure of TT-specific Abs in serum and fecal samples by ELISA and passive cutaneous anaphylaxis

Antibody titers in serum and fecal samples were determined by ELISA as described previously (28, 29). Falcon (Microtest III) microtiter plates (Becton Dickinson, Oxnard, CA) were coated with a 100-μl aliquot of 5 μg/ml of TT or CT-B (Sigma Chemical Co.) in PBS. Wells were blocked with PBS-0.05% Tween (PBS-T) containing 10% normal goat serum. Serial twofold dilutions of serum (starting at 1/16) or fecal extracts (starting at 1/4) were added to wells in duplicate and incubated for 4 h at room temperature. Detection Abs consisted of peroxidase-labeled goat anti-mouse μ-, α-, and γ-chain-specific Abs (1 μg/ml) (Southern Biotechnology Associates, Birmingham, AL) and the chromogenic substrate, ABTS with H_2O_2 (Moss, Inc., Pasadena, MD), was added to individual wells for color development. For IgG subclass determination, biotinylated mAbs specific for IgG1 (2 μg/ml), IgG2a (1 μg/ml), IgG2b (0.5 μg/ml), and IgG3 (1 μg/ml; PharMingen, San Diego, CA) and streptavidin-conjugated peroxidase were employed. End-point titers were expressed as the reciprocal $\log_2$ of the last dilution that gave an optical density of 0.2 OD units (414 nm) greater than those values obtained with serum or fecal samples from nonimmunized mice after incubation for 30 min. The passive cutaneous anaphylaxis test was performed to measure TT-specific IgE Abs in serum as previously described (29). Fisher rats were sensitized by injecting threefold dilutions of mouse serum into the shaved back. After 16 h, TT in 1% Evan's blue dye in PBS was injected i.v. Bluing at the site of serum injection represented a positive reaction.

B cell ELISPOT

The methods for isolating PP, SP, and intestinal laminal propria mononuclear cells were previously described in detail (28–31). Cells were resuspended in complete medium (RPMI 1640, Cellgro Mediatech, Washington DC) containing 10% FCS, HEPES buffer (15 mM), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). Ag-specific and total IgM, IgA, and IgG spot-forming cells (SFC) were enumerated in cell suspensions by TT-specific ELISPOT as described previously (28, 29, 32). Briefly, 96-well nitrocellulose-based plates were coated with 5 μg/ml of TT or CT-B diluted in PBS for the enumeration of Ag-specific SFC, and with 2 μg/ml of rat monoclonal anti-mouse μ, γ or α Ab (Southern Biotechnology Associates) diluted in PBS for examination of total SFC. Control wells received PBS only. Wells were blocked with 5% FCS-PBS. Serial 10-fold dilutions starting at 10^6 cells were added to these wells in duplicate and incubated for 6 h. SFC were detected with peroxidase-labeled anti-mouse μ, γ or α Ab (1 μg/ml; Southern Biotechnology Associates) and then visualized by adding the chromogenic substrate, 3-amino-9-ethylcarbazole (Moss, Inc.). Spots were counted with the aid of a dissecting microscope (SZH Zoom Stereo Microscope System, Olympus, Lake Success, NY).

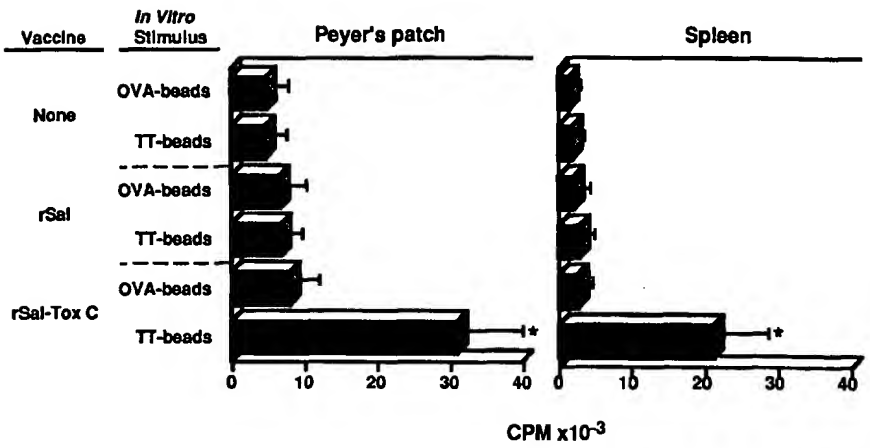

FIGURE 1. Induction of Ag-specific $CD4^+$ T cells by oral immunization of mice with r*Salmonella* BRD 847 expressing Tox C. $CD4^+$ T cells were isolated from PP and SP of mice orally immunized with 5×10^9 CFU or r*Salmonella* BRD 847 expressing Tox C or r*Salmonella* BRD 509 (no expression of Tox C) or nonimmunized (None). $CD4^+$ T cells (2×10^6/ml) were cultured with TT- or OVA-coated beads (20 beads/cell) in the presence of APC (1×10^6/ml) and IL-2 (10 U/ml) for 4 days. During the last 16 h of incubation, 0.5 μCi [^{3}H]thymidine/well was added. The amount of ^{3}H incorporation was determined by scintillation counts. The data shown are from 4 to 6 wk after immunization; results are from five mice per group and are expressed as the mean ± SEM of five different experiments. *, $p < 0.01$ vs OVA-coated beads.

Antigen-induced $CD4^+$ T cell proliferation

$CD4^+$ T cells from nonadherent SP and PP cell suspensions were purified by the magnetic activated cell sorter system (Stefen Miltenyi Biotechnologic Equipment, Bergish-Gladbach, Germany) as previously described (25). Cells were passed through the magnetized column after incubation with biotinylated anti-L3T4 (GK 1.5) and streptavidin-conjugated microbeads. This procedure yielded enriched $CD4^+$ T cell preparations of >95%. For the stimulation of Ag-specific $CD4^+$ T cells in vitro, Ag was adsorbed to latex microspheres as described previously (23, 29, 33). $CD4^+$ T cells were restimulated in vitro according to previously described methods (23, 29). $CD4^+$ T cells (2×10^6 cells/ml) were cultured with rIL-2 (10 U/ml, PharMingen), and T cell-depleted, irradiated feeder spleen cells from naive mice in flat-bottom 96-well (100 μl/well) or 24-well (1 ml/well) tissue culture plates (Corning Glass Works, Corning, NY) for proliferation and cytokine analysis, respectively. To measure proliferation, 0.5 μCi of tritiated [^{3}H]thymidine (Amersham Corp., Arlington Heights, IL) was added after 3 days of culture and 16 h before harvest. Approximately 20 Ag-coated particles/cell were found to optimally stimulate $CD4^+$ T cell cultures based on the extent of cellular proliferation (Fig. 1), levels of cytokines in culture supernatants, and numbers of specific cytokine SFC. Control wells consisted of cells only or cells incubated with unabsorbed beads or irrelevant protein-coated beads. All cell cultures were maintained at 37°C in a 5% CO_2 incubator.

Cytokine ELISA

Cytokine levels in culture supernatants were determined by ELISA using the mAbs listed in Table I. Falcon Microtest III plates (Becton, Dickinson) were coated with 100 μl of anti-cytokine Ab diluted in PBS and incubated overnight at 4°C. The wells were blocked with PBS-T containing 1% BSA at room temperature for 1 h. Serial twofold dilutions of supernatants were added to duplicate wells and incubated overnight at 4°C. The wells were washed with PBS-T and incubated with the appropriate biotinylated anti-cytokine mAb diluted in PBS-T with 1% BSA for 1 to 2 h. Following three rinses, wells were incubated with peroxidase-labeled anti-biotin Ab (0.5 μg/ml; Vector Laboratories, Inc., Burlingame, CA) for 1 h and developed with ABTS containing H_2O_2 (Moss, Inc.). Standard curves were generated using murine rIFN-γ, rIL-5, rIL-6, and rIL-10 (Genzyme, Cambridge, MA); rIL-2 (PharMingen); and rIL-4 (Endogen, Boston, MA). Background was determined for each cytokine assay by substituting different recombinant cytokines as the only change. The ELISA assays were capable of detecting 0.78 U/ml of IFN-γ and IL-5, 0.20 U/ml of IL-2, 20 pg/ml of IL-4 and IL-10, and 4 U/ml of IL-6. For statistical analysis, levels of cytokine below the detection limit were recorded as one-half the detection limit (e.g., IFN-γ = 0.39 U/ml).

Cytokine ELISPOT

The cytokine-specific ELISPOT assays for the detection of murine IFN-γ, IL-2, IL-4, IL-5, and IL-6 were modified from our previous studies (31, 34, 35). To minimize background spots, concentrations of anti-cytokine mAbs (Table I) and peroxidase-conjugated Abs were sequentially reduced until pseudospots had disappeared in control wells. A variety of control wells were employed, including 1) coating with PBS instead of an anti-cytokine Ab, 2) coating with a different anti-cytokine Ab, 3) substituting a different biotinylated secondary Ab, 4) adding 50 μg/ml cycloheximide during the cell incubation stage to inhibit de novo cytokine synthesis, and 5) using unstimulated and Con A-activated spleen cells from unimmunized mice. The optimal concentrations of coating and biotinylated secondary mAbs used in this study are listed in Table I. For all assays, 96-well nitrocellulose-based microtiter plates (Millititer HA, Millipore Corp., Bedford, MA) were coated overnight at 4°C with 100 μl of the appropriate anti-cytokine mAb diluted in PBS. After removing the coating solutions from the plate, wells were blocked for 2 h at room temperature in PBS containing 5% FCS and then rinsed with PBS. The cells were added to individual wells (5×10^5, 5×10^4, and 5×10^3 cells/100-μl well) and incubated for 16 to 20 h at 37°C in a humidified, 5% CO_2 incubator. Wells were rinsed extensively in PBS containing PBS-T and incubated at room temperature for 2 h in specific biotinylated anti-cytokine Ab diluted with PBS-T containing 5% FCS. Following rinsing with PBS-T, peroxidase-labeled anti-biotin Ab (0.8 μg/ml) diluted in PBS-T with 5% FCS was added to wells. Plates were incubated at room temperature for 30 min, and spots were visualized with the substrate 3-amino-9-ethylcarbazole (Moss, Inc.) as described above

Table I. *Reagents used for cytokine ELISA and ELISPOT assays*

	Monoclonal Antibodies[a]			
	Coating (rat anti-mouse)		Detection (Biotin-rat anti-mouse)	
Cytokine Assay	Designation (clone)	Concentration (μg/ml)	Designation (clone)	Concentration (μg/ml)
IFN-γ ELISA	R4-6A2	10.0	XMG1.2	0.3
ELISPOT	R4-6A2	5.0	XMG1.2	0.1–0.5
IL-2 ELISA	JES6-1A12	5.0	JES6-5H4	0.4
ELISPOT	JES6-1A12	2.5	JES6-5H4	0.2–0.4
IL-4 ELISA	BVD4-1D11	2.0	BVD6-24G2	0.2
ELISPOT	BVD4-1D11	2.5	BVD6-24G2	0.1–0.25
IL-5 ELISA	TRFK-5	10.0	TRFK-4	4.0
ELISPOT	TRFK-5	5.0	TRFK-4	0.2–0.5
IL-6 ELISA	MP5-20F3	2.0	MP5-32C11	0.5
ELISPOT	MP5-20F3	2.5	MP5-32C11	0.2–0.5
IL-10 ELISA	JES5-2A5	2.0	SXC-1	0.3

[a] All mAbs were obtained from PharMingen except mAb R4-6A2 (Lee Biomolecular, San Diego, CA).

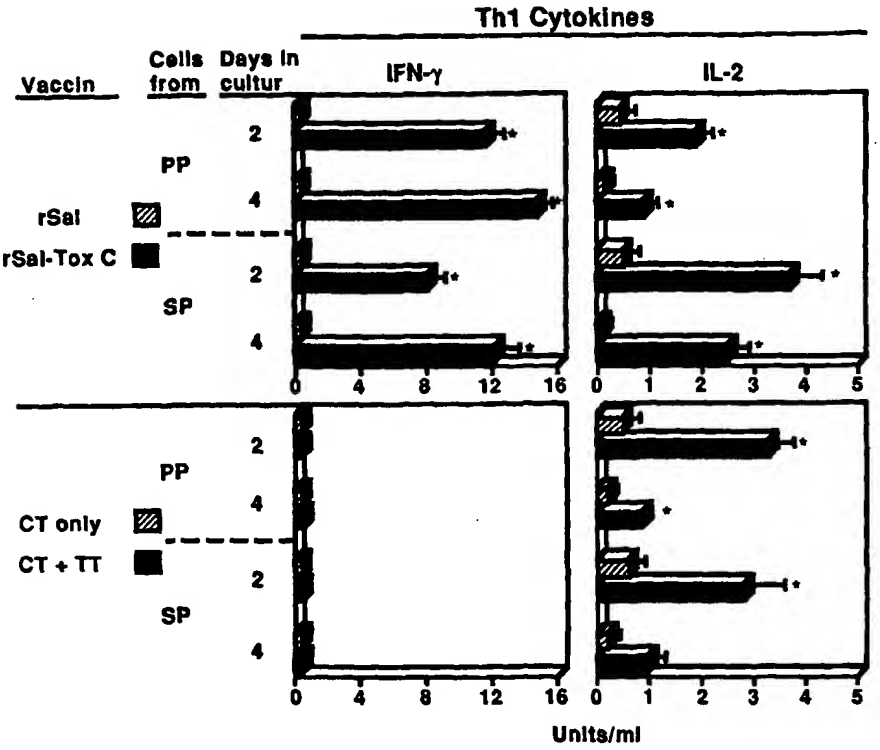

FIGURE 2. Profile of IFN-γ and IL-2 synthesis by $CD4^+$ T cells isolated from mice orally immunized with *rSalmonella* BRD 847 expressing Tox C. To examine levels of IFN-γ and IL-2 synthesis, PP and SP $CD4^+$ T cells isolated from mice orally immunized with a single dose of *rSalmonella* BRD 847 expressing Tox C (5×10^9 CFU) or three doses of TT (250 μg) and CT (10 μg) were cultured with TT- or OVA-coated latex microspheres in the presence of APC. Control mice were given BRD 509 or CT only. Culture supernatants were harvested following 2 or 4 days of incubation and then analyzed by the respective cytokine-specific ELISA. OVA-containing cultures had no detectable IFN-γ, <0.78 U/ml of IL-2 on day 2, and <0.2 U/ml of IL-2 on day 4. The data shown are 4 to 6 wk after immunization; results are from five mice per group and are expressed as the mean ± SEM of six different experiments. *, $p < 0.01$ vs control mice.

(see *B cell ELISPOT*). Background numbers of spots observed in OVA-containing control cultures ranged from 10 to 20 cells/10^6 cells for IL-2, IL-4, and IL-6 and <1 cell/10^6 cells for IFN-γ and IL-5.

Capillary electrophoresis of cytokine-specific RT-PCR products

PP and SP $CD4^+$ T cells were restimulated with TT as described above, but purified by removal of adherent cells and by negative panning on petri plates coated with goat $F(ab')_2$ anti-mouse Ig. A CD4-enriched T cell subset was obtained by complement-mediated lysis of $CD8^+$ T cells using mAb 2.43 (American Type Culture Collection, Rockville, MD) and complement. Following 2 days of incubation, restimulated cells were sorted by flow cytometry into $CD4^+$ Mac-1^- T cells and Mac-1^+ $CD4^-$ cells using FITC-conjugated anti-L3T4 mAb and biotinylated anti-CD11b (Mac-1) Abs and PE-conjugated streptavidin (PharMingen). Similar cell populations were obtained from cultures of restimulated whole mononuclear cell suspensions. Total mRNA was isolated from Ag-restimulated cells with Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) and reverse transcribed with Superscript RT (Promega, Madison, WI). RT of approximately 300 ng of RNA was performed. The cDNA was then diluted 1/15 to 1/30 for PCR amplification of β-actin and 1/4 to 1/8 for PCR amplification of specific cytokine. These RNA amounts and dilutions were based on obtaining a linear relationship between input RNA and PCR product. Levels of amplified cDNA for selected cytokines (IFN-γ, IL-4, IL-5, IL-6, and IL-10) were determined by capillary electrophoresis (P/ACE 5000, Beckman Instruments, Fullerton, CA) (36, 37). Amplified cDNA samples were separated in a coated capillary (internal diameter, 100 μm; 37 cm) filled with Tris-borate-EDTA containing replaceable linear polyacrylamide (Beckman Instruments). Samples (10 μl) were diluted with double distilled H_2O to a total volume of 100 μl. Injections (7.8 nl) were conducted hydrodynamically for 10 s at 0.5 psi. The separations were performed in the same buffer containing DNA fluorescent stain molecules (0.4 μg/ml) and run at 200 V/cm for 25 min. All cytokine values were normalized to the corresponding β-actin values.

Statistics

The results are expressed as the mean ± SEM. Statistical significance ($p < 0.05$) was analyzed by Student's *t* test and by ANOVA followed by Fisher least significant difference test.

Results

$CD4^+$ Th cell cytokine patterns

To determine the pattern of Th cytokine production induced to Tox C in mice orally immunized with BRD 847, it was first necessary to standardize culture conditions for optimal stimulation of T cells in vitro. $CD4^+$ T cells were prepared from mice orally immunized 4 to 6 wk previously with BRD 847. In preliminary studies, we showed that the kinetics of r*Salmonella* BRD 847 colonization in PP, MLN, and SP were similar in BALB/c and C57BL/6 mice (data not shown). We used the C57BL/6 strain in all subsequent studies, since these mice are also high responders to the oral immunogen and adjuvant CT. Maximal T cell proliferation was achieved when $CD4^+$ T cells from PP or SP (2×10^6/ml) were incubated with 1×10^6 feeder cells/ml, 10 U/ml of rIL-2, and 20 TT-coated latex microspheres/$CD4^+$ T cell (Fig. 1). Cells incubated without Ag or with OVA-coated microspheres demonstrated reduced stimulation, as did cells from BRD 509 immune mice incubated with TT-coated beads.

Profiles of Th1 and Th2 cytokine production were elucidated in PP and SP from mice orally immunized with a single dose of BRD 847 expressing Tox C. Initially, culture supernatants from TT-stimulated PP and SP $CD4^+$ T cells were examined for Th1 cytokine synthesis (e.g., IFN-γ and IL-2) by ELISA. For comparative purposes, parallel experiments were conducted with $CD4^+$ T cells isolated from mice orally immunized with TT in another form, as a soluble protein together with CT as adjuvant. In this regard, only orally administered r*Salmonella*-Tox C induced IFN-γ production by PP and SP $CD4^+$ T cells (Fig. 2). In contrast, $CD4^+$ T cells obtained from mice orally immunized with TT and CT did not produce detectable levels of IFN-γ, consistent with our previous studies (23, 29). Both forms of TT delivery resulted in the rapid appearance of IL-2 in TT-stimulated $CD4^+$ T cell cultures. The kinetics of the appearance of these cytokines were distinct, with

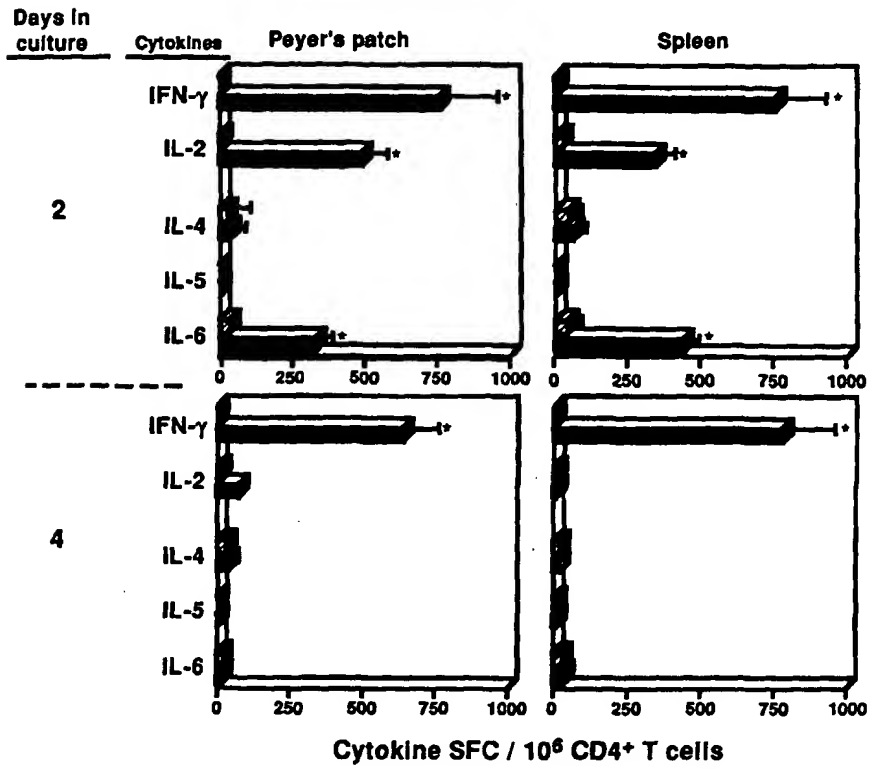

FIGURE 3. Frequency of Th1 and Th2 cytokine-producing cells in TT-stimulated $CD4^+$ T cells isolated from PP and SP of orally immunized mice. PP and SP $CD4^+$ T cells from mice orally immunized either with BRD 847 expressing Tox C (solid bars) or with control BRD 509 (hatched bars) as adjuvant were isolated and cultured as described in Figure 1. $CD4^+$ T cells were harvested from in vitro cultures following 2 or 4 days of incubation. The numbers of TT-specific IFN-γ-, IL-2-, IL-4-, IL-5-, and IL-6-producing cells were examined by individual cytokine-specific ELISPOT assay. The data shown are 4 to 6 wk after immunization; results are from five mice per group and are expressed as the mean ± SEM of three different experiments. *, $p < 0.01$.

IL-2 appearing soon and decreasing by day 4, while IFN-γ was increased in supernatants on day 4 (Fig. 2).

To enumerate cytokine-producing cells, $CD4^+$ T cells were isolated from the TT-stimulated cultures and were then examined by IFN-γ- and IL-2-specific ELISPOT assays. Th1-type cells were dominant in mice orally immunized with BRD 847 expressing Tox C, since PP and SP $CD4^+$ T cells from these mice contained high numbers of IFN-γ (760 ± 200 SFC/10^6 cells) and IL-2 SFC (505 ± 100 SFC/10^6 cells; Fig. 3). It should be noted that elevated numbers of IFN-γ SFC were seen throughout the culture period. In contrast, increased numbers of IL-2 SFC were noted only on day 2, and few IL-2 SFC were seen by day 4 in mice orally immunized with BRD 847 expressing Tox C. The profiles of these cytokine-producing PP and SP $CD4^+$ T cells correlated with the levels of cytokine measured in culture supernatants (Figs. 2 and 3).

These results were confirmed by RT-PCR analysis of IFN-γ transcripts. For this analysis, $CD4^+$ T cells were repurified following 2 days of incubation by specific staining followed by FACS sorting to remove contaminating APCs. The RNA isolated from both PP and SP $CD4^+$ T cells showed increased levels of IFN-γ mRNA in cells from TT-stimulated cultures compared with OVA-stimulated controls (Table II). Collectively, these findings showed that oral administration of *rSalmonella* expressing Tox C induced Ag-specific Th1-type responses in both mucosal and systemic compartments.

Oral immunization of attenuated rS. typhimurium *elicits DTH responses*

TT-specific DTH responsiveness was assessed after oral immunization with r*S. typhimurium* vaccine strains BRD 509, BRD 753, and BRD 847. A single dose of BRD 847 or BRD 753 elicited TT-specific DTH responses. Higher DTH responses were observed in mice orally immunized with BRD 847 than in mice orally immunized with BRD 753 (Fig. 4). Injection of TT into the ears of BRD 509 immune mice resulted in minimal or background ear swelling (Fig. 4). These findings further confirm that oral administration of BRD 847 expressing Tox C induced TT-specific Th1 type cells that mediate DTH responses.

Oral rSalmonella *induce CD4⁺ Th1-type cells*

It is known that intracellular microorganisms, including *Salmonella*, generally induce cytokines that are characteristic of Th1-type responses in vivo (8–12). To this end, analysis of Th2 cytokine synthesis by TT-stimulated PP and SP $CD4^+$ T cells from mice orally immunized with BRD 847 expressing Tox C revealed that neither IL-4 nor IL-5 was detected in the culture supernatants (Fig. 5*A*). This finding suggested that Th2-type responses were not induced in mucosal or systemic compartments by oral immunization with r*S. typhimurium* BRD 847 expressing Tox C. In contrast, orally administered TT and CT induced Ag-specific Th2 type $CD4^+$ T cells, since high levels of secreted IL-4 and IL-5 were noted in culture supernatants from TT-stimulated PP and SP $CD4^+$ T cell cultures (Fig. 5*A*) (23). Taken together, it was shown that orally administered r*Salmonella* expressing Tox C did not induce IL-4- or IL-5-secreting Th2-type responses in either PP or SP, while TT and CT induced Th2-type cells that produced both IL-4 and IL-5.

This view was further supported by cytokine-specific ELISPOT and RT-PCR analyses. Thus, few IL-4 and IL-5 SFC were evident in both TT-stimulated PP and SP $CD4^+$ T cells from mice orally immunized with r*Salmonella* expressing Tox C (Fig. 3). For RT-PCR analysis of IL-4 and IL-5 message, $CD4^+$ T cells were repurified following 2 days of incubation, as described above, for the evaluation of IFN-γ message. No specific elevation in IL-4 or IL-5 message was present in SP or PP $CD4^+$ T cells (Table II). These results indicated that orally administered r*Salmonella* vaccine

Table II. *RT-PCR analysis of mRNA in CD4⁺ T cells and Mac-1⁺ cells from mice orally immunized with rSalmonella-Tox C*

Tissue Source	Cultured Cells[a]	Sorted Cells[b] for RNA Extraction	Relative Peak Area (β-actin = 100)[c] IFN-γ OVA	IFN-γ TT	IL-4/IL-5 OVA	IL-4/IL-5 TT	IL-6[d] OVA	IL-6[d] TT	IL-10 OVA	IL-10 TT
SP	Whole cells	$CD4^+$	24	71	<2	<2			<2	20
		$Mac\text{-}1^+$	10	68	<2	<2	9	261	8	52
	$CD4^+$	$CD4^+$	<2	32	<2	<2			<2	10
		$Mac\text{-}1^+$	4	21	<2	<2	10	45	4	15
PP	Whole cells	$CD4^+$	18	85	<2	<2			3	18
		$Mac\text{-}1^+$	15	65	<2	<2	22	162	6	28
	$CD4^+$	$CD4^+$	5	18	<2	<2			<2	6
		$Mac\text{-}1^+$	6	15	<2	<2	2	10	3	8

[a] Cells were cultured as total mononuclear cells or as $CD4^+$ T cells purified by negative selection and incubated with antigen-presenting cells and rIL-2 in the presence of antigens (TT or OVA coated beads).

[b] Cells from 2-day cultures were sorted by flow cytometry into $CD4^+$ $Mac\text{-}1^-$ T cells and $Mac\text{-}1^+$ $CD4^-$ cells.

[c] Cytokine-specific RT-PCR was performed under strictly defined conditions. For each sample, the final results were recorded as the % increase in cytokine-specific message relative to β-actin (= 100) according to the peak areas obtained by using capillary electrophoresis. Results are representative of two experiments conducted.

[d] Results for IL-6 mRNA in $CD4^+$ cells were highly variable and therefore could not be conclusively determined.

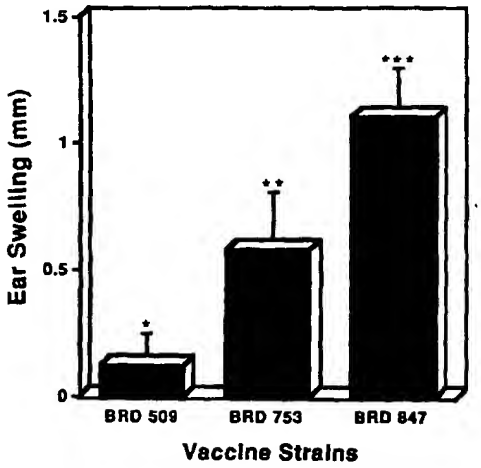

FIGURE 4. Induction of Ag-specific DTH responses by oral immunization of mice with r*Salmonella* BRD 847 expressing Tox C. For the assessment of cell-mediated immune responses, 30 μg of TT or PBS was injected into the left or right ear pinna, respectively, of mice orally immunized with BRD 847 (stable expression of Tox C), BRD 753 (unstable expression of Tox C), or BRD 509 (no expression of Tox C). Ear swelling was measured 24 h later with a spring-loaded measuring caliper. ***, >; **, >; *, $p < 0.01$ (by ANOVA followed by Fisher least significant difference test).

failed to preferentially expand typical Th2-type T cells that produce IL-4 and IL-5.

Oral rSalmonella expressing Tox C induces IL-10 in $CD4^+$ Th cells and IL-6 in macrophages

Although SP and PP $CD4^+$ T cells from mice immunized with r*Salmonella* expressing Tox C produced no IL-4 or IL-5 (Fig. 5*A*), these cultures contained cells producing both IL-6 and IL-10 (Fig. 5*B*). Thus, 10 to 60 U/ml of IL-6 and 10 to 50 pg/ml of IL-10 were detected in supernatants from TT restimulated SP and PP $CD4^+$ T cell cultures. The kinetics of IL-6 and IL-10 accumulation in the culture supernatants differed, with IL-6 levels remaining constant after day 2 while IL-10 increased in supernatants from day 2 to day 4 (Fig. 5*B*). In addition, although IL-6 SFC were detected on day 2, few were observed by day 4 (Fig. 3).

Since IL-6 and IL-10 are also produced by non-T cells (e.g., macrophages), it was important to ensure that $CD4^+$ T cells were responsible for the IL-6 and IL-10 gene expression observed in mice immunized with r*Salmonella* expressing Tox C. To this end, $CD4^+$ Mac^- T cells and $Mac\text{-}1^+$ $CD4^-$ cells were sorted by flow cytometry from day 2 cultures. Since cytokine-secreting cells were greatly diminished after cell sorting, RT-PCR was employed for the characterization of RNA extracts. Capillary electrophoresis of cDNA products revealed that only IL-10 message was consistently elevated in SP and PP $CD4^+$ T cells restimulated with TT, whereas levels of both IL-6 and IL-10 were elevated in $Mac\text{-}1^+$ cells (Table II). Whole mononuclear cell suspensions restimulated and sorted into $CD4^+$ T cell and $Mac\text{-}1^+$ cell populations showed the same pattern of IL-6 and IL-10 gene expression in the two cell populations. Interestingly, IFN-γ, but not IL-4 or IL-5, message was also elevated in $Mac\text{-}1^+$ cells, probably reflecting the presence of activated NK cells (Table II). These findings provided evidence that oral r*Salmonella* vaccine delivery facilitates the induction of predominant Ag-specific Th1-type IFN-γ-producing cells and Th2 cells producing IL-10 as well as increased IL-6 production by macrophages in mucosal inductive sites in the gastrointestinal (GI) tract (PP) and in systemic tissue (SP).

Mucosal and systemic anti-TT Ab responses in mice orally immunized with BRD 847 rSalmonella expressing Tox C

Inasmuch as orally administered BRD 847 expressing Tox C induced predominant Th1 responses with IL-10 presumably produced by Th2 cells, it was of interest to examine the isotype and subclass of TT-specific Ab synthesis. To this end, serum and fecal samples were obtained weekly following oral immunization, and TT-specific Ab titers were determined by ELISA. Maximum serum IgG responses were obtained 1 mo after oral immunization (Fig. 6*A*) and remained high throughout the study (up to 8 wk). When Ag-specific serum IgG subclasses were examined, high IgG2a Ab titers were noted, followed by IgG2b, IgG1, and IgG3 (Fig. 6*B*). In addition, IgE Ab responses were not detected in sera to TT (Table III). This pattern of TT-specific IgG subclass responses reflects the help provided by Th1-type cells in mice orally immunized with r*Salmonella*. In contrast, oral immunization with TT plus CT adjuvant resulted in predominant IgG1 Ab titers (14.2 ± 0.6), followed by IgG2b (8.4 ± 1.2), IgG2a (4.5 ± 0.5), and IgG3 (<4); IgE Abs were also detected in serum (Table III) (29).

Furthermore, Ag-specific IgA responses, which have been shown to correlate with the expansion of Th2 cells (24–26), were

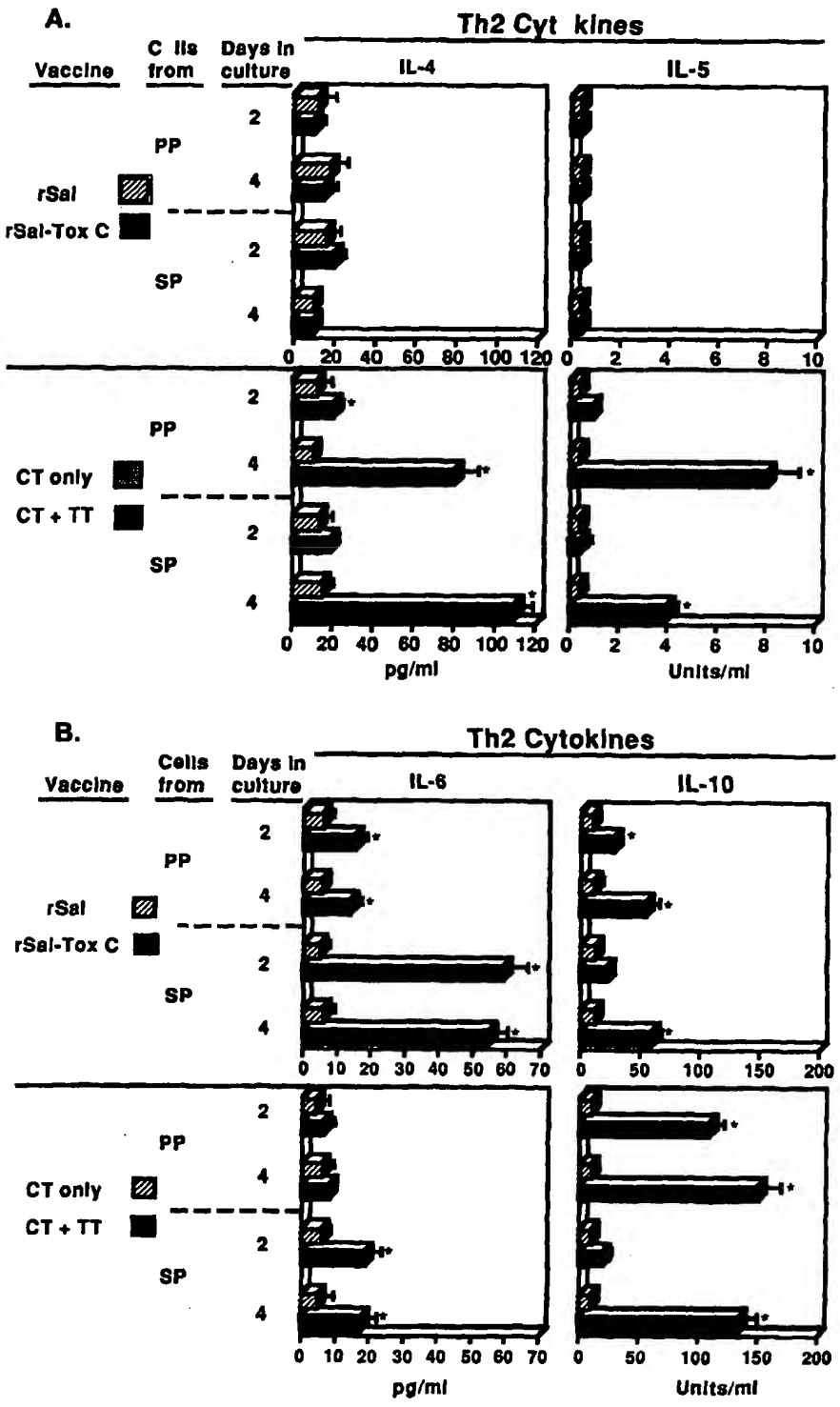

FIGURE 5. Analysis of Th2 cytokine (IL-4, IL-5, IL-6, and IL-10) synthesis by $CD4^+$ *T cells isolated from PP and SP of* mice orally immunized with *rSalmonella* BRD 847 expressing Tox C or with TT plus CT. Culture supernatants were obtained and evaluated as described in Figure 1 for the elucidation of secreted IL-4 and IL-5 (*A*) as well as IL-6 and IL-10 (*B*) by using respective cytokine-specific ELISA. OVA-containing cultures had no detectable IL-4, IL-5, or IL-10 and 4 to 6 U/ml of IL-6.

elevated in both serum and fecal samples of mice orally immunized with *rSalmonella* (Figs. 6*A* and 7*A*). Since high titers of TT-specific IgA Ab were seen in both serum and intestinal secretions, it was important to establish the origin of the Ag-specific fecal IgA response. To do this, intestinal lamina propria lymphocytes from immunized mice were examined by Ag- and isotype-specific ELISPOT assay. Significant numbers of TT-specific SFC were observed 1 mo after oral immunization (Fig. 7*B*). These numbers remained high throughout the 8-wk test period. The predominant SFC response was of the IgA isotype in the lamina propria lymphocytes, where approximately 150 to 200 IgA-SFC/10^6 cells were TT specific. This frequency as well as fecal IgA titers (1/128) were similar to those observed in mice orally immunized with TT plus CT (Table II) (25, 28). Fewer IgA anti-TT SFC were present in the PP of orally immunized mice (Fig. 7B), a finding consistent with this tissue serving as an inductive and not an effector site.

Table III. *Summary of TT-specific Th cell responses for orally immunized mice*

Vaccine	Antibody Responses[a]			DTH	T Helper Cell Responses[b]				
	Mucosal compartment	Systemic compartment			Dominant Th response	Type 2 cytokines			
	IgA	IgG	IgE[c]			IL-4	IL-5	IL-6	IL-10
							CD4$^+$ Th cells		
TT + CT	+	G1 ≥ G2a	+	n.d.[d]	Th2 (IL-4)	+	+	+	+
							CD4$^+$ Th cells		
r*Salmonella*-Tox C	+	G2a ≥ G1	−	+	Th1 (IFN-γ)	−	−	±	+
							Mac-1$^+$ cells[e]		
						−	−	+	+

[a] Mucosal IgA responses to TT were measured in fecal samples by ELISA and in lamina propria cell suspensions by ELISPOT. TT-specific serum IgG responses were measured by ELISA and serum IgE responses were measured by PCA.

[b] Th cell responses are representative of PP and SP CD4$^+$ T cell suspensions restimulated in vitro with TT antigen. Results were obtained using cytokine-specific ELISA, ELISPOT, and RT-PCR assays. DTH was measured by the ear swelling method. A '−' signifies levels below detection.

[c] A '+' indicates a serum TT-specific IgE titer of >1:270; a '−' indicates that TT-specific IgE was not detected at a starting serum dilution of 1:10.

[d] n.d. = Not determined.

[e] Cytokine-specific gene expression in Mac-1$^+$ CD4$^-$ cells were determined by RT-PCR followed by capillary electrophoresis. Mac-1$^+$ cells are enriched in macrophages and NK cells.

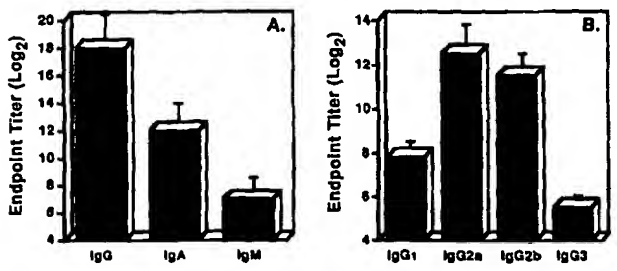

FIGURE 6. Isotype and subclass analysis of TT-specific Ab responses in serum of mice orally immunized with r*Salmonella* BRD 847 expressing Tox C. Isotypes of Ag-specific serum Ab (*A*) and IgG subclass (*B*) responses were examined in these orally immunized mice using Ag-, isotype-, and IgG subclass-specific ELISA. TT-specific Ab responses were not detected in sera from mice orally immunized with BRD 509 (beginning at 1/16).

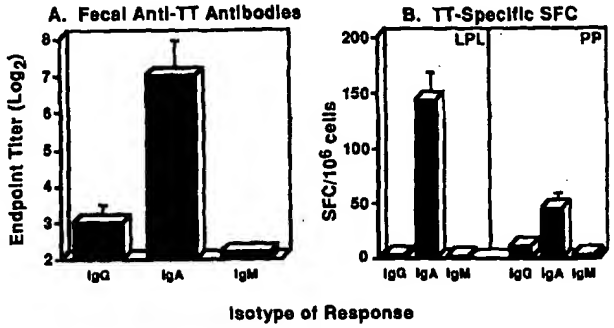

FIGURE 7. Induction of TT-specific IgA responses by oral immunization with r*Salmonella* BRD 847 expressing Tox C. Fecal extracts from orally immunized mice were examined to determine the level of Ag-specific IgA Abs (*A*). To confirm local production of TT-specific IgA Ab in mucosal effector sites, intestinal laminal propria (LP) mononuclear cells were isolated from these orally immunized mice and then examined by ELISPOT assays (*B*). The data represent the mean value (±SEM) from five groups of five mice. TT-specific Ab responses were not detected following oral immunization with BRD 509.

Discussion

In this study, we have shown that two different mucosal Ag delivery protocols resulted in the selective induction of distinct CD4$^+$ T cell subset responses, and for convenience, these results are summarized in Table III. Oral delivery of attenuated r*S. typhimurium* vector expressing Tox C induced mainly TT-specific Th1-type cells producing IFN-γ and IL-2 in mucosa-associated (PP) and systemic (SP) tissues accompanied by increases in distinct Th2 cytokines, IL-6 and IL-10, but not IL-4 or IL-5 (Table III). Predominant induction of Th1-type responses was supported by the presence of DTH in vivo (Fig. 4) and high serum titers of TT-specific IgG2a Abs in mice orally immunized with BRD 847 expressing Tox C (Table III). In support of our results, it has been shown that IFN-γ derived from Th1 cells largely supports IgG2a synthesis (38). In contrast, mice that received TT and CT via the oral route harbored a dominant Th2-type response characterized by increased IL-4, IL-5, and IL-6 synthesis (Figs. 2 and 5), a finding consistent with our previous results (23, 29). In the present study, we found that orally administered TT and CT resulted in elevated Th2-type IL-10 secretion as well. Thus, elevated TT-specific IgG1 responses were noted in mice orally immunized with TT and CT (29). These results demonstrated that oral administration of a protein vaccine in two different delivery systems (e.g., r*Salmonella*-Tox C or TT mixed with the mucosal adjuvant CT) induced two distinct Th1 and Th2 cell response profiles, which probably influenced the outcome of serum Ag-specific IgG subclass responses (e.g., IgG2a vs IgG1), respectively (Table III). Further, although there is some debate as to the degree to which CT induces Th1 or Th2 activity to coadministered protein (23, 29, 38, 39), the present study clearly shows that relative to the ability of r*Salmonella*-Tox C to enhance TT-specific Th1 cells, the adjuvant effect of CT for enhancing Th1 cells to TT is essentially negative.

Support of our conclusions showing that BRD 847 and CT plus TT vaccines induce distinct Th cell responses was presented in a recent paper (40). In this study, fluid secretion was measured in intestinal loops of mice immunized with *S. typhimurium* (strain TML-R66) or CT. Early (18 h) and late responses (18 days) to *Salmonella* were dominated by IFN-γ without IL-4. In contrast, IFN-γ was not detected in intestinal loops of mice given CT at early and late time points, but IL-4 was detected.

Ag-specific IgA responses were induced in both mucosal and systemic compartments of mice orally immunized with r*Salmonella* BRD 847 expressing Tox C (Figs. 6*A* and 7). The levels of these Ag-specific IgA responses were comparable to those induced by oral immunization with TT and CT, an effective mucosal vaccine regimen for induction of Ag-specific Th2-type responses and enhanced TT-specific IgA Ab synthesis (23, 28). Our past work has shown that Th2-type cells and their derived cytokines (e.g., IL-5 and IL-6) were essential for the induction of IgA B cell responses (19, 20, 23). However, our present findings have provided new evidence that Ag-specific IgA responses can proceed efficiently in both mucosal and systemic compartments during a dominant Th1 cell response. Thus, we have shown that high TT-specific IgA responses were induced in the GI tract following induction of IFN-γ- and IL-2-producing Th1 cells and in the absence of IL-4- and IL-5-secreting Th2 cells.

Induction of TT-specific IgA B cell responses in a Th1-dominant environment could be explained by the existence of alternate Th1 and Th2 cell pathways. One obvious possibility is that IL-2 secreted by Th1 cells may provide a replacement activation signal for the induction of IgA B cell responses. To this end, it was shown that IL-2 can support limited IgA synthesis (6). However, the IgA-enhancing ability of IL-2 was less efficient compared with that of IL-5 or IL-6. Both IL-5 and IL-6 have been shown to be the most effective cytokines that promote the differentiation of IgA-committed B cells to become IgA-producing plasma cells (6, 19–21).

The inefficiency of Th1 cells for promoting IgA secretion in vivo may be overcome, provided an alternative source of Th2 cytokine is available within the microenvironment of the interacting cells. To this end, our results showed that macrophages producing high levels of IL-6 were found in PP and SP of mice orally immunized with r*Salmonella* expressing Tox C. Indeed, *Salmonella* have a predilection for macrophages that are abundant in the GI tract. Since we did not detect Th2 cells producing IL-4 and IL-5, these macrophages producing IL-6 along with Ag-specific Th2 cells producing IL-10 could enhance the proliferation and secretion of limiting numbers of Ag-specific post-switch IgA-positive cells for high IgA production. In fact, IL-6 has been shown to be the most effective terminal differentiation factor for IgA-committed B cells to become IgA-producing cells in both human and mouse systems (6, 19–21). In IL-6 gene-disrupted mice, mucosal IgA responses were markedly reduced and could be restored by mucosal immunization with a vector producing IL-6 (41), thereby demonstrating directly in vivo that a source of IL-6 other than Th2 cells could fully restore mucosal IgA responses. Thus, macrophages producing IL-6 and Th2 cells producing IL-10 may together enhance IgA secretion by plasma cells entering mucosal effector sites in the absence of preferential expansion of classical Th2 cells producing IL-4, IL-5, and IL-6.

Our results also suggest that compensatory Th2-type cells are induced in mice orally immunized with r*Salmonella*, and Th2 cells producing IL-10 may become major helper types for mucosal IgA responses together with adjacent macrophages producing IL-6. In this regard, our results showed that IL-10-producing $CD4^+$ T cells could be considered as second level Th2 cells that support the mucosal IgA response even in the presence of a dominant Th1-type response. Although IL-6 has been shown to be a major cytokine for murine IgA responses (20, 41), IL-10 until now has only been associated with human IgA responses (42). It will be necessary to isolate clones of mouse $CD4^+$ T cells that secrete IL-10 to determine their potential role in the regulation of mucosal IgA responses. Studies along these lines are currently underway.

Unlike IL-2, IFN-γ fails to stimulate enhanced IgA responses in vitro (17, 20). This finding was supported in vivo by our recent observation that TT-specific mucosal IgA responses were rapidly induced in mice lacking a functional IFN-γ gene and immunized with the same r*Salmonella* Tox C vaccine (J. L. VanCott et al., manuscript in preparation). Moreover, mucosal IgA responses were suppressed in the lungs of mice immunized with fowlpox virus expressing IFN-γ compared with the control vector alone (43). Thus, IFN-γ production by Th1 cells in mice immunized with r*Salmonella* Tox C probably plays only a small role in the development of mucosal IgA responses. However, IFN-γ production plays a critical role in host resistance to *Salmonella* species by activating macrophages for cell killing (44).

According to the results of the present investigation, we propose that at least two mechanisms exist for the induction and maintenance of mucosal IgA responses. For example, oral administration of TT and CT preferentially activates a Th2 cell pathway that leads to the induction of IL-4, IL-5, IL-6, and IL-10 for the generation of high IgA B cell responses in both mucosa-associated and systemic tissues. This requirement for Th2 cell induction was evidenced by the finding that oral immunization of IL-4 gene-disrupted mice with TT or other soluble proteins (i.e., keyhole limpet hemocyanin or OVA) with CT as adjuvant failed to elicit mucosal IgA Ab responses (45). On the other hand, oral immunization of normal mice with live vector, e.g., r*Salmonella* expressing Tox C, induced mainly Th1 cells producing IFN-γ and IL-2 along with IL-10-producing Th2-type cells and high level IL-6 production by macrophages. This second or Th1-inducing vaccine regimen also elicited mucosal IgA responses. Further evidence that this latter vaccine affected IgA production via a different mechanism was suggested by a separate study in which oral administration of this vaccine to IL-4 gene-disrupted mice readily elicited mucosal IgA responses (Okahashi, et al., manuscript submitted for publication).[4] Therefore, the existence of alternative mechanisms for IgA induction could explain how Ag-specific IgA B cell responses are maintained in the presence of dominant Th1- vs Th2-type cell responses.

Our results showing a distinct pattern of Th1 and Th2 cell responses in systemic as well as mucosal tissues of mice orally immunized with r*Salmonella* expressing Tox C are consistent with recent reports describing Th cell responses to other intracellular bacteria (46, 47). For example, immunization of mice with *Brucella abortus* induced Th1-like IFN-γ gene expression and Th2-like IL-10 gene expression. As in the present study, specific IL-6 production by $CD4^+$ T cells could not be conclusively demonstrated. The increase in systemic IL-10 was proposed to function by limiting inflammatory reactions that often accompany immune responses to intracellular bacteria. However, it is possible that IL-10 could also play a role in the development of IgA responses, as has been found in humans (42). We cannot conclude, however, whether IL-10 was produced by Th1 cells or a novel Th2 cell subset secreting only IL-10, although it has been shown that IL-10 can be expressed by either IFN-γ- or IL-4-producing Th cells (48).

Although we found a predominance of Th1-type cells in both IgA-inductive (e.g., PP) and systemic (e.g., SP) tissues of mice orally immunized with r*Salmonella* expressing Tox C, it is possible that Ag-specific Th1 and Th2 cells in IgA effector sites (e.g., intestinal lamina propria) produce distinct sets of cytokines that promote IgA responses. Th1, Th2, and uncommitted Th cells arising from the PP of orally immunized mice could express different cytokines upon migration to mucosal effector sites and possibly switch from a Th1 to a Th2 type. It has been shown that Th1 cells expressing only IL-2 can be driven to a Th1 or Th2 cell type by appropriate signaling (49). Thus, an appropriate signal could be generated due to the unique environment of mucosal effector tissues for PP-derived Th1 cells or Th2 cells producing only IL-10 to

become a Th2 type capable of producing IL-5 and IL-6. This possible preferential expansion of Th2 cells in effector sites would correlate with the finding that the intestinal lamina propria is especially enriched in Th2-type IL-5-secreting cells (31). Thus, increased Ag-specific IgA responses would be induced and maintained in mice orally immunized with r*Salmonella*.

Our results have shown that the use of two different oral delivery systems allows the selective manipulation of Th cell and B cell immune responses to the vaccine Ag TT. Further, it was shown that strong mucosal IgA responses can arise when dominant Th1 or Th2 responses are generated in PP-inductive sites of the GI tract (Table III). We are currently generating T cell clones to further characterize cytokine profiles of Th1 and Th2 cells associated with the two different Ag delivery protocols used in this study.

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